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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C07K 14/46, 7/08, A61K 38/17, 38/10

A2

(11) International Publication Number: WO 00/09553

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18575

(22) International Filing Date: 13 August 1999 (13.08.99)

(30) Priority Data:
60/096,607 14 August 1998 (14.08.98) US

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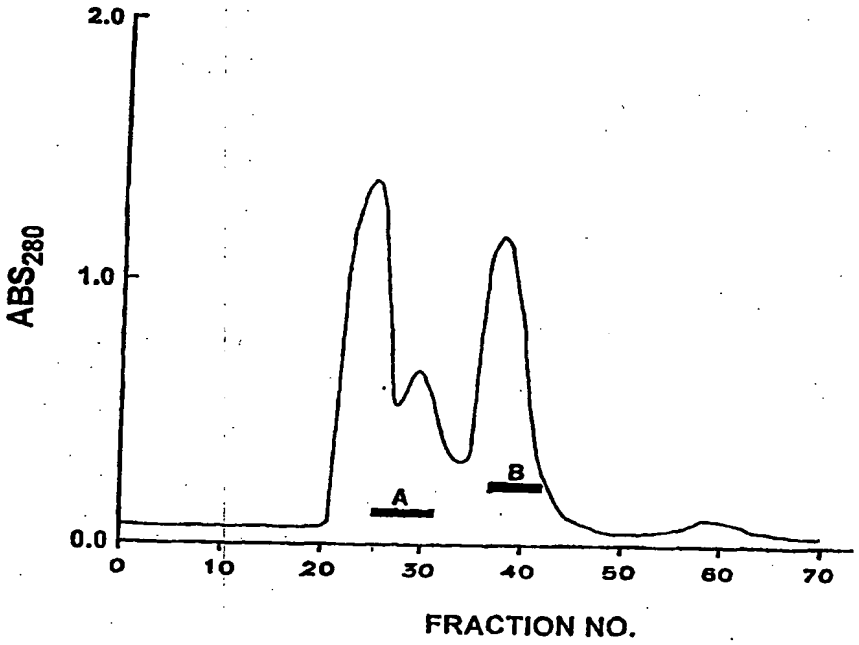
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ANTIMICROBIAL PEPTIDES ISOLATED FROM THE SKIN OF AMERICAN FROGS



(57) Abstract

The invention is directed to 7 families of Rana peptides isolated from the skin of 6 species of American frogs, as well as modified and truncated versions thereof. These peptides are termed Rana peptides and have antibacterial activity.

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ANTIMICROBIAL PEPTIDES ISOLATED FROM THE SKIN OF AMERICAN FROGS

This application is based on U.S. Provisional Application Serial No. 60/096,607, filed August 14, 1998.

Background of the Invention

The indiscriminate use of antibiotics in hospitals has created drug-resistant pathogens that cause difficult-to-cure infections. This problem is particularly serious in the case of AIDS, tuberculosis and other immunocompromised patients. For example, data collected by the Synercid Microbiology Assessment of Resistance Trends surveillance project have shown that more than 31% of 17,000 bacterial isolates of *Streptococcus pneumoniae* obtained from patients at U.S. hospitals were intermediately or completely resistant to penicillin. The incidence of methicillin-resistant strains of *Staphylococcus aureus* was shown by this project to be 29%. Tuberculosis, which was allegedly eradicated in the United States, has made a horrific reappearance in forms that are resistant to traditional isoniazid therapy. Bacterial infection in animals has also become drug-resistant in some cases. The economic toll is staggering as well. It has been estimated that drug-resistant infections acquired in hospitals nearly triple the cost and duration of an average hospital stay. Hoffert, S.P., "Companies seeking solutions to emerging drug resistance," *The Scientist,* **12(8)**, 1-6 (1998).

As a result of earlier dramatic successes in the development of antimicrobial agents, most large pharmaceutical companies during the 1980s and early 1990s cut back or eliminated programs of research and development of new compounds on the grounds that the battle against infectious diseases had been won. However, the emergence of increasing numbers of pathogenic microorganisms with resistance to the commonly used antibiotics has greatly stimulated searches for novel antimicrobial agents to fight drug-resistant infections.

Among those searches is the investigation of novel antibiotic peptides from Anuran (frog and toad) skin. Amphibians of necessity live in a warm, moist environment that is particularly conducive to the growth of microorganisms. As a result, Anurans have evolutionarily developed effective strategies for their own protection.

In particular, Anurans synthesize polypeptides with a broad spectrum of antimicrobial activity through the granular glands present in their skin. The bioactive peptides are released into skin secretions in a holocrine fashion upon stress or injury and protect against invasion by pathogenic microorganisms. The amphibian antimicrobial peptides are generally synthesized as members of structurally-related families and examples include magainins from *Xenopus laevis*, bombinins from *Bombina variegata* and *Bombina orientalis*, dermaseptins from *Phyllomedusa sauvagii* and *Phyllomedusa bicolor*, buforins from *Bufo bufo gargarizans*, and caerins from *Litoria chloris* and *Litoria splendida*. Despite the sequence similarities, the members of a particular family have distinct spectra of antimicrobial activity and it has been speculated that this molecular diversity is important in protecting the animal from invasion by a wide array of different microorganisms.

Frogs from the genus *Rana* constitute an extremely diverse and widely distributed group with an estimated 250 species worldwide and at least 36 species having been identified in North America (12 delete). Analysis of skin secretions and/or skin extracts of different species of Ranid frogs has led to the following characterizations: 1) gaegurins and rugosins from *R. rugosa*, 2) brevinins from *R. brevipoda porsa*, *R. esculenta* and *R. sphenocephala*, 3) esculentins from *R. esculenta*, 4) ranalexin and ranatuerins from *R. catesbeiana* and 5) temporins and ranatuerin 1T from *R. temporaria*. Peptides of the brevinin family have also been isolated from an extract of gastric tissue from *R. esculenta*. Further examples of such peptides are discussed in:

1) Lazarus, L.H., and Attila, M., "The toad, ugly and venomous, wears yet a precious jewel in his skin," *Prog. Neurobiol.*, 41, 473-507 (1993);

2) Zasloff, M., "Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms and partial cDNA sequence of a precursor," *Proc. Natl. Acad. Sci.* USA 84, 5449-5453, (1987);

3) Buforins from *Bufo bufo gargarizans*- Park, C.B., Kim, M.S., and Kim, S.C., *Biochem. Biophys. Res. Commun.*, 218, 408-413, (1996);

4) Bombinins from *Bombina variegata*- Simmaco, M., Barra, D., Chiarini, F., Noviello, L., Melchiorri, P., Kreil, G., and Richter, K., "A family of bombinin-related peptides from the skin of *Bombina variegata," Eur. J. Biochem.* 199, 217-222, (1991);

5) *Bombina orientalis*- Gibson, B.W., Tang, D., Mandrell, R., Kelly, M., and Spindel, E.R., "Bombinin-like peptides with antimicrobial activity from skin secretions of the Asian toad, *Bombina orientalis.*," *J. Biol. Chem.* 266, 23103-23111, (1991);

6) Dermaseptins from *Phyllomedusa sauvagii*- Mor, A., Hani, K., and Nicolas, P., "The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms," *J. Biol. Chem.* 269, 31635-31641, (1994);

7) Caerins from *Litoria chloris*- Steinborner, S.T., Currie, G.J., Bowie, J.H., Wallace, J.C., and Tyler, M.J., "New antibiotic caerin 1 peptides from the skin secretion of the Australian tree frog *Litoria chloris*, comparison of the activities of the caerin 1 peptides from the genus *Litoria*," *J. Peptide Res.* 51, 121-126, (1998);

8) Gaegurins from the Korean frog, *Rana rugosa*- Park, J.M., Jung, J.E., and Lee, B.J., "Antimicrobial peptides from the skin of a Korean frog, *Rana rugosa*," *Biochem. Biophys. Res. Commun.* 205, 948-954, (1994);

9) Brevinins from the Japanese frog, *Rana brevipoda porsa*- Morikawa, N., Hagiwara, K., and Nakajima, T., "Brevinin-1 and -2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*," *Biochem. Biophys. Res. Commun.* **189**, 184-190 (1992); and

10) Esculentins and brevinins from the European frog, *Rana esculenta*- Simmaco, M., Mignogna, G., Barra, D., and Bossa, F., "Antimicrobial peptides from skin secretions of Rana esculenta," *J. Biol. Chem.* **269**, 11956-11961, (1994).

Some of these peptides discussed in the art have the following sequences.

Gaegurin 5	FLGALFKVASKVLPSVKCAITKKC	(SEQ ID NO: 1)
Gaegurin 6	FLPLLAGLAANFLPTIICKISYKC	(SEQ ID NO: 2)
Brevinin 1	FLPVLAGIAAKVVPALFCKITKKC	(SEQ ID NO: 3)
Brevinin 1E	FLPLLAGLAANFLPKIFCKITRKC	(SEQ ID NO: 4)
Peptide A1	FLPAIAGILSQLF	(SEQ ID NO: 5)
Peptide B9	FLPLIAGLLGKLF	(SEQ ID NO: 6)

To date, no antibacterial peptides have been isolated from the adult American bullfrog and studies of immature bullfrogs have indicated the presence of only one antibacterial peptide, ranalexin having the sequence: FLGGLIKIVPAMICAVTKKC (SEQ ID NO: 7).

The present invention has as its goal the isolation and characterization of amphibian antimicrobial peptides to assess their value as taxonomic and phylogenetic markers. A further goal of the present invention is the study of seven different classes of Anuran peptides with antimicrobial activity which are obtained from extracts of the skins of six closely related species of North

American frogs of the genus *Rana:* the spotted frog *R. luteiventris,* the Rio Grande leopard frog *R. berlandieri,* green frog R. clamitans, the pig frog R. grylio. and the northern leopard frog *R. pipiens* and the North American bullfrog, R *catesbeiana*.

Summary of the Invention

These and other objects are achieved by the present invention which is directed to seven families of peptides obtained from North American frogs (families of Rana peptides), to purified Rana compositions containing these peptides, to purified antibacterial extracts from Rana skin, to modified Rana peptides, to truncated Rana peptides, to pharmaceutical compositions containing Rana peptides or modified forms thereof, and to methods for treatment of bacterial infections using Rana peptides or modified forms thereof.

In particular, the invention is directed to seven families of Rana peptides shown below in Table 1. These Rana peptides have antibacterial activity and are selected from any of the following seven peptide families. The Rana peptides may be in the form of their C-terminus carboxylic acids, or modified as C-terminus amides or C-terminus esters. The amides may be a simple amide (CONH2), which is the isolated form for the Temporin-A and –B Families, or amides derived from C1 to C10 primary, secondary or tertiary aliphatic or aromatic amines. The esters (COOR wherein the R group is the alcohol residue) are derived from C1 to C10 aliphatic or aromatic alcohols. The preferred C-terminus forms for the Temporin-A and Temporin-B families are the simple amides.

In these formulas, the single letters designate amino acid residues according to accepted convention except that in the formulas for the peptides designated with a Family name followed by the term "mod", the letters X, B, Z, U, O, J' and J, which are not conventional single letter designations of amino acid residues, are used as symbols to indicate a substitution of either any of the amino acid residues occurring at the same position within the other peptides of

the same family or a conservative substitution of an amino acid residue for the amino acid substitution of an amino acid residue at the same position for any of the peptides within the same family of peptides. These symbols for the Family-mod formulas have two identifiers: first is the identity of the symbol itself (X, B, Z, U, O, J' or J); second is the location/position of the symbol within the formula. For example, X of the Ranatuerin-1 mod appears at the 3, 16 and 21 positions of the formula. At each of these positions, X has a different meaning depending upon the corresponding amino acid residues at the same position of the other members of the family. In the case of the Ranatuerin-1 mod formula, the X means that at the 3 position, the amino acid residue can be L, I or a conservative substitution therefor, at the 16 position the amino acid residue can be L, F or a conservative substitution therefor, and at the 21 position the amino acid residue can be I, V or a conservative substitution therefor.

The term conservative substitution means substitution of an amino acid residue by another that has the same side chain ionicity, basicity, acidity, lipophilicity or hydrogen bonding character as the residue being replaced. Examples include isoleucine (I), leucine (L), alanine (A), valine (V), phenyl analine (F), proline (P) and glycine (G) as an interchangeable group; lysine (K), histidine (H) and arginine (R) as an interchangeable group, serine (S), tyrosine (Y) and threonine (T) as an interchangeable group; cysteine (C) and methionine (M)as an interchangeable group asparagine (N), glutamine (Q) and tryptophan (W) as an interchangeable group, and aspartic acid (D) and glutamic acid (E) as an interchangeable group. The letters after these amino acids are their single letter symbols.

The dashes in the ranatuerin-2 family peptides provide alignment of homologous peptide residues. The single and multiple dashes indicate a bond between the two amino acid residues on either side of the space occupied by the dashes. For the purpose of determining amino acid substitutions for this family, each dashed line constitutes a spacer instead of an amino acid residue. For example, the 5 dashed lines of Ranatuerin-2 stand in the place of 5 amino acid

residues so that the A residue following the dashed lines is counted in the 19th position for the purpose of placing the amino acid residues of the individual peptides of the family into homologous alignment. Likewise, the 4 and 8 dashed lines of the Ranatuerin-2Ca and Ranatuerin-2Cb peptides respectively place the A residue following those lines into the 19th position.

TABLE 1

Families of Rana Peptides

Ranatuerin-1 family		
Ranatuerin-1	SMLSVLKNLGKVGLGLVACKINKQC	(SEQ ID NO: 8)
Ranatuerin-1G	SMISVLKNLGKVGLGFVACKVNKQC	(SEQ ID NO: 9)
Ranatuerin-1C	SMLSVLKNLGKVGLGLVACKINKQC	(SEQ ID NO: 10)
Ranatuerin-1 mod	SMXSVLKNLGKVGLGXVACKXNKQC	(SEQ ID NO: 11)
Ranatuerin-2 family		
Ranatuerin-2	GLFLDTLKGAAKD-----AGK-LEGLKCKITGCKLP	(SEQ ID NO: 12)
Ranatuerin-3	GFL-DIIKNLGKTF----AGHMLDKIKCTIGTCPPSP	(SEQ ID NO: 13)
Ranatuerin-2P	GLM-DTVKNVAKNL----AGHMLDKLKCKITGC	(SEQ ID NO: 14)
Ranatuerin-2B	GLL-DTIKGVAKTV----AASMLDKLKCKISGC	(SEQ ID NO: 15)
Ranatuerin-2Ca	GLFLDTLKGAAKDV----AGKLLEGLKCKIAGCKP	(SEQ ID NO: 16)
Ranatuerin-2Cb	GLFLDTLKGL--------AGKLLQGLKCIKAGCKP	(SEQ ID NO: 17)
Ranatuerin-2La	GI-LDSFKGVAKGVAKDLAGKLLDKLKCKITGC	(SEQ ID NO: 18)
Ranatuerin-2Lb	GILSSI-KGVAKGVAKNVAAQLLDTLKCKITGC	(SEQ ID NO: 19)
Ranatuerin-2G	GLLLDTLKGAAKDI----AGIALEKLKCKITGCKP	(SEQ ID NO: 20)
Ranatuerin-2 mod	GBBBBBBKBBBBBBBBBBABBBBBBBKCBBBBCBBB	(SEQ ID NO: 21)
Esculentin-2 family		
Esculentin-2L	GILSLFTGGIKALGKTLFKMAGKAGAEHLACKATNQC	(SEQ ID NO: 22)
Esculentin-2B	GLFSILRGAAKFASKGLGKDLTKLGVDLVACKISKQC	(SEQ ID NO: 23)
Esculentin-2P	GFSSIFRGVAKFASKGLGKDLARLGVNLVACKISKQC	(SEQ ID NO: 24)
Esculentin-2 mod	GZZSZZZGZZKZZZKZLZKZZZZZGZZZZACKZZZQC	(SEQ ID NO: 25)
Brevinin-1 family		
Ranatuerin-4	FLPFIARLAAKVFPSIICSVTKKC	(SEQ ID NO: 26)

Brevinin-lLa	FLPMLAGLAASMVPKLVCLITKKC	(SEQ ID NO: 27)
Brevinin-lLb	FLPMLAGLAASMVPKFVCLITKKC	(SEQ ID NO: 28)
Brevinin-lBa	FLPAIAGMAAKFLPKIFCAISKKC	(SEQ ID NO: 29)
Brevinin-lBb	FLPAIAGMAAKFLPKIFCAISKKC	(SEQ ID NO: 30)
Brevinin-lBc	FLPFIAGVAAKFLPKIFCAISKKC	(SEQ ID NO: 31)
Brevinin-lBd	FLPAIAGVAAKFLPKIFCAISKKC	(SEQ ID NO: 32)
Brevinin-lBe	FLPAIVGAAAKFLPKIFCVISKKC	(SEQ ID NO: 33)
Brevinin-lBf	FLPFIAGMAANFLPKIFCAISKKC	(SEQ ID NO: 34)
Brevinin-lPa	FLPIIAGVAAKVFPKIFCAISKKC	(SEQ ID NO: 35)
Brevinin-lPb	FLPIIAGIAAKVFPKIFCAISKKC	(SEQ ID NO: 36)
Brevinin-lPc	FLPIIASVAAKVFSKIFCAISKKC	(SEQ ID NO: 37)
Brevinin-lPd	FLPIIASVAANVFSKIFCAISKKC	(SEQ ID NO: 38)
Brevinin-lPe	FLPIIASVAAKVFPKIFCAISKKC	(SEQ ID NO: 39)
Brevinin-1 mod	FLPUIUGUAAUUUPUUUCUIUKKC	(SEQ ID NO: 40)

Ranalexin family

Ranalexin-1	GFLGGLMKAFPALICAVTKKC	(SEQ ID NO: 41)
Ranalexin-1	CFLGGLMKIIPAAFCAVTKKC	(SEQ ID NO: 42)
Ranalexin-1 mod	FLGGLMKJ'J'PAJ'J'CAVTKKC	(SEQ ID NO: 43)

Temporin A family as the C-terminus carboxamide

Ranatuerin 5	FLPIASLLGKYL	(SEQ ID NO: 44)
Ranatuerin 6	FISAIASMLGKFL	(SEQ ID NO: 45)
Ranatuerin 7	FLSAIASMLGKFL	(SEQ ID NO: 46)
Ranatuerin 8	FISAIASFLGKFL	(SEQ ID NO: 47)
Ranatuerin 9	FLFPLITSFLSKVL	(SEQ ID NO: 48)
Temporin-lCa	FLPFLATLLSKVL	(SEQ ID NO: 49)
Temporin-lCb	FLPFLAKILTGVL	(SEQ ID NO: 50)
Temporin-lCc	FLPLFASLIGKKL	(SEQ ID NO: 51)
Temporin-lPa	FLPIVGKLLSGLL	(SEQ ID NO: 52)
Temporin-lLc	FLPILINLIHKGL	(SEQ ID NO: 53)
Temporin-A mod	FOOOOOOOOOOOLO	(SEQ ID NO: 54)

Temporin B Family as the C-terminus carboxamide

Temporin-lLa	VLPLISMALGKLL	(SEQ ID NO: 55)
Temporin-lLb	NFLGTLINLAKKIM	(SEQ ID NO: 56)
Temporin-lGa	SILPTIVSFLSKFV	(SEQ ID NO: 57)
Temporin-lGb	SILPTIVSFLSKFL	(SEQ ID NO: 58)
Temporin-lGc	SILPTIVSFLTKFIL	(SEQ ID NO: 59)
Temporin-lGd	FIIPLIASFLSKFIL	(SEQ ID NO: 60)
Temporin-B mod	JJJJJIJSFLJKJJL	(SEQ ID NO: 61)

The purified compositions of the invention includes any composition containing one or more of the foregoing peptides obtained from a natural source such as Rana skin or obtained from a synthetic source.

The modified Rana peptides of the invention include any of the foregoing Rana peptides designated as Ranatuerin-1 mod, Ranatuerin-2 mod, Esculentin-2 mod, Brevinin-1 mod, Brevinin-2 mod, Temporin-A mod and Temporin-B mod wherein the symbols X, B, Z, U, O, J' and J indicate substitutions of amino acid residues as discussed above. The modified Rana peptides of the invention also include the C-terminus amides and esters described above, which are derived from C1 to C10 aliphatic or aromatic amines or alcohols. Further modified Rana peptides include those wherein one or more of the amino acid residues valine (V), alanine (A), lysine (K), and glutamic acid (E) are substituted for the glycine (G) residues. Further modified Rana peptides of the invention include any of the foregoing Rana peptides wherein arginine (R) is substituted for one or more of the lysine (K) residues.

Additional modified Rana peptides contain modified side chains so that any of the Rana peptides with amino acid residues having functionalized side chains can be modified with amidation or esterification groups. Examples include those in which the epsilon amino group of a lysine (K) residue has been coupled to a C_{10} to C_{20} fatty acid such a palmitate or glycosylated sugar, those in which the epsilon carboxy group of aspartic (D) or glutamic (E) acid residues or hydrolyzed glutamine (Q) residue have been coupled to fatty alcohols or glycosylated sugars, or those in which the epsilon hydroxyl group of threonine (T), tyrosine (Y), or serine (S) residues have been coupled to fatty acids or glycosylated sugars. In particular, ranatuerin 1 having any of these side chain

modifications, where the appropriate amino acid residue is present, is preferred. In addition, the modified Rana peptides include those in which a truncated fragment is synthetically condensed with a non-natural peptide or peptide sequence optionally containing heterocyclic organic moieties such as a beta or gamma amino acid, an aliphatic diamine, an aliphatic or aromatic dicarboxylic acid, pyridine carboxylic acid, aromatic diols and the like.

The truncated versions of Rana peptides include the fragments formed by cleaving at any of the glycine residues or between any repeat residues, and fragments formed by N-terminal deletion of pairs of amino acid residues. Further truncated versions include those that are cleaved at the single or multiple dash symbols. For example, truncated versions of ranatuerin 1 include those containing amino acid residues 1-9, the fragment containing amino acid residues 16 - 25, N-terminally deleted fragments such as ranatuerin 1 (3-25), (5-25), (7-25), (9-25), (11-25), (13-25), (15-25), (17-25) and (19-25).

The pharmaceutical composition of the invention includes the combination of one or more Rana peptides with a pharmaceutical carrier rendering the composition suitable for an appropriate route of administration to a patient.

The method of treatment of the invention involves administration of an effective amount of the pharmaceutical composition by an appropriate route to a patient suffering from a target bacterial infection.

Brief Description of the Drawings

FIG. 1 presents a graph of a gel permeation chromatography of an extract of the skin of Rana *luteiventris*.

FIGS. 2A, B present graphs of a reverse-phase HPLC of the antimicrobial activity of R. *luteiventris* skin.

FIGS. 3A, B present graphs of the chromatographic results of a partial purification of the fractions from R. *luteiventris* skin having antimicrobial activity.

FIGS. 4A, B and C present graphs of the chromatographic results of a purification of ranatuerin 2La, ranatuerin 2Lb and esculentin 2L.

FIG. 5 presents gel permeation chromatography on Sephadex G-25 of an extract of the skin of *Rana catesbeiana* after partial purification on Sep-Pak cartridges. The fractions in zone A contained ranatuerins 1-5 and the fractions in zone B contained ranatuerins 6-9.

FIGS. 6A and 6B present reversed-phase HPLC on a semipreparative Vydac C-18 column. FIG. 6A presents the pooled fractions from zone A in Fig. 5 and Fig. 6B presents the pooled fractions from zone B in FIG. 5. The dashed line shows the concentration of acetronitrile in the eluting solvent and the bars show the fractions containing the ranatuerins indicated.

Detailed Description of the Invention

The present invention is directed to a systematic investigation of extracts of the skin of adult Ranid frogs and characterization of all the peptides with inhibitory activity towards bacteria. The skins of frogs of the genus *Rana* produce a diverse array of peptides with antimicrobial activity. A comparison of the amino acid sequences of these peptides demonstrates that they may be arranged in families on the basis of structural similarity. Attempts to catalogue the antimicrobial peptides are hampered by the absence of a systematic terminology and by the fact that peptides clearly belonging to the same structurally family have been given different names. As shown in Table 1, Summary of the Invention, at least 7 different peptide families produced in the skins of Ranid frogs have been identified on the basis of amino acid sequence similarity. For the present invention, these families are named from the first member to have been discovered as follows: (1) ranatuerin-1, (2) ranatuerin-2 which includes ranatuerin-3, (3) brevinin-1 which includes gaegurin-5 and -6, and ranatuerin-4, (4) brevinin-2 which includes rugosin A, and B and ranatuerin-IT, (5) esculentin-2 which includes gaegurin-l, -2, -3 and -4 and rugosin C, (6) ranalexin and (7) temporin which includes peptides Al and B9 and ranatuerins -5, -6, -7, -8 and -9.

The C-terminal region of peptides of the brevinin-1, ranalexin, brevinin-2, esculentin-1, esculentin-2 and ranatuerin-1 families contains a cystine-bridged cyclic lieptapeptide whereas the corresponding region of peptides of the ranatuerin-2 family contains a cystine-bridged cyclic hexapeptide. The temporin family peptides lack the cyclic region and their primary structures have been poorly conserved. All members of the family contain between 10-14 amino acid residues and terminate in a C-terminally (α-amidated amino acid residue.

The present invention establishes that extracts of the skins of six closely-related North American frogs of the genus *Rana* contain antimicrobial peptides belonging to 5 of the known families: brevinin-1, ranatuerin-2, esculentin-2, ranalexin and temporin. These peptides classified by using the initial letter of the species name to indicate their origin (L for *luteiventris,* B for *berlandieri* and P for *pipiens,* C for *clamitans* and G for *grylio.* The *catesbeiana* peptides are regarded as the "parent" and so are not given a letter.) Where two or more members of the same family are produced by the one species, the peptides are differentiated by the letters a, b, c, etc. as in the examples of ranatuerin-2La and ranatuerin-2Lb.

The distribution of the molecular forms of the biological active peptides and the primary structures of the individual peptides are highly species-specific. Each frog in this study produced multiple peptides of the brevinin-1 family and at least one peptide of the esculetin-2 and ranatuerin-2 families but a peptide of the temporin family was not detected in *R. berlandieri* skin despite a systematic search for such a component. Similarly, peptides that are structurally related to ranalexin and ranatuerin-1, previously isolated from the North American bullfrog, *R. catesbeiana*, were not identified in the skin extracts of the other species. At this time, peptides belonging to the esculentin-1 and brevinin-2 families have not been identified in the skins of North American Ranid frogs. None of the antimicrobial peptides isolated in this study have been described previously.

It has been proposed that the synthesis of multiple structurally related peptides by the skin of an amphibian species serves to protect the organism against invasion by a broad array of microorganisms because each peptide has a discrete spectrum of antimicrobial activity and may target specific bacteria. The peptides of the brevinin-1, esculentin-2 and ranatuerin-2 families isolated in the present study show a broad spectrum of antimicrobial activity inhibiting the growth of a gram positive bacterium *(Staphylococcus aureus),* a gram negative bacterium *(Escherichia coli)* and a yeast *(Candida albicans).* However, of the microorganisms tested, the peptides of the temporin family were active only against *S. aureus.*

As shown in Table 1, Summary of the Invention, ranatuerin 4 contains limited structural similarity, including the presence of a C-terminal heptapeptide ring, to the previously characterized gaegurins (Park, J.M., Jung, J.E., and Lee, B.J., *Biochem. Biophys. Res. Commun.* **205**, 948-954 (1994)); and brevinins (Morikawa, N., Hagiwara, K., and Nakajima, T., *Biochem. Biophys. Res. Commun.* **189**, 184-190 (1992); Simmaco, M., Mignogna, G., Barra, D., and Bossa, F., *J. Biol. Chem.* **269**, 11956-11961 (1994)) also isolated from the skin of other Ranid frogs. Ranatuerins 5 - 9 show some sequence identity to the hemolytic peptides A1 and B9 previously isolated from the skin of *Rana esculenta* (Simmaco, M., De Biase, D., Severinin, C., Aita, M., Falconieri Erspamer, G., Barra, D., and Bossa, F., *Biochim. Biophys. Acta* **1033**, 318-323 (1990)). Ranatuerins 1, 2, and 3, however, do not resemble closely any other anti-microbial peptides isolated from amphibian skin.

The amino acid sequence motif C-K-[V/I/L]-[A/S/T]-K-[K/T/Q]-C, asserted for all antimicrobial peptides from species of the genus *Rana* without exception (Park, J.M., Jung, J.E., and Lee, B.J., *Biochem. Biophys. Res. Commun.* **205**, 948-954 (1994)), is absent from the many of the Rana peptides of the present invention. A further unexpected feature of the present invention is that ranalexin, previously isolated from an extract of whole bullfrog tadpoles (Clark, D.P., Durell, S., Maloy, W.L., and Zasloff, M., *J. Biol. Chem.* **269**,

10849-10855 (1994)), is not present among the ranatuerins. Since ranalexin shows potent cytolytic activity towards *S. aureus* (minimal inhibitory concentration of 4 μg/ml), it would not have been missed during the investigation of the bullfrog skin extracts. It is probable, therefore, that the expression of the antimicrobial peptides in bullfrog skin is developmentally regulated.

Many of the Rana peptides show high potency and a broad spectrum of antimicrobial properties against the gram positive, gram negative bacteria and against the fungi, see the tables and figures. Thus, the amino acid sequence of the potent Rana peptides can also form the basis for modified versions exhibiting higher potency and with specificity towards pathogenic microorganisms that have developed resistance to commonly used antibiotics.

In particular, one modification focuses upon the presence of a cystine bridge at the C-terminal end of the brevinin-1, ranalexin, brevinin-2, esculentin-1, esculentin-2 and ranatuerin-1 families and the ranatuerin-2 family. Synthesis of modified Rana peptides containing the substitutions Cys → Ala and Cys → Ser provides peptides that will have no cysteine bridges. These modified Rana peptides will show inhibitor activity against *Escherichia coli, Staphylococcus aureus* and *Candida albicans.*

In general, other modified ranatuerins can be formulated through an understanding of the mechanism by which antimicrobial peptides are cytotoxic. Antimicrobial peptides such as the Rana peptides have a propensity to form amphipathic α-helical structures and their mechanism of action is believed to involve a direct interaction with the fatty acyl chains in the cell membrane leading to disruption of normal membrane function responsible for osmotic balance. The presence of cationic residues (particularly lysine) in antimicrobial peptides is believed to destroy the ionic gradient across cell membranes by forming ion channels. Cruciani, R.A., Barker, J.L., Durell, S.R., Raghunathan, G., Guy, H., Zasloff, M., and Stanley, F., *Eur. J. Pharmacol.* 226, 287-296 (1992). Analysis of the secondary structure of a Rana peptide, either by the

method of Garnier, Osguthorpe and Robson or by the method of Qian and Sejnowski, predicts that most of the peptides contain two α-helical regions joined by a glycine-containing "hinge" region. Garnier, J., Osguthorpe, D., and Robson, B., *J.Mol. Biol.* **120**, 97-121 (1978); Qian, N., and Sejnowski, T., *J. Mol. Biol.* **202**, 865-884 (1988)). This conformation is, for example, found in the cecropin family of antimicrobial peptides isolated from insect hemolymph and from mammalian intestine.

Garnier, Osguthorpe and Robson

SMLSVLKNLGKVGLGFVACKINKQC (SEQ ID NO: 62)
AAAAAAA-TBBBBBBBAAATTTTTT

Qian and Sejnowski

SMLSVLKNLGKVGLGFVACKINKQC (SEQ ID NO: 63)
--AAAAA--------AAAA-----

A α-helix; B β-sheet; T β-turn; - random coil

Based upon this understanding, one generation of modified Rana peptides is constructed by substituting one or more of the α-helix-extending residues (hydrogen bonding residues) for the lipophilic amino acid residues within the helix regions of an individual Rana peptide, especially those within the C-terminus region of the peptides. Other amino acid residues can be substituted as follows:

(a) Ala is substituted for Gly in order to replace the β-sheet region in the central portion of the peptide with an extended α-helix. The increased α-helical character of the peptide promotes interaction with the cell membrane of the targeted microorganism;

(b) Val is substituted for Gly in order to stabilize the β-sheet region in the central portion of the peptide;

(c) Ala or another lipophilic amino acid residue is substituted for Asn in order to promote α-helical character;

(d) Glu is substituted for Gly in order to promote α-helical character.

Modified Rana peptides also include those Rana peptide Family modified peptides with the formulas containing the symbols X, B, Z, U, O, J' and J as discussed in the Summary of the Invention.

Still further modified Rana peptides can also prepared by substituting one or more of the lysine residues by arginine. This substitution will increase the cationic character of the peptide so as to promote its ability to affect the cellular membrane ionic gradient.

Another generation of modified Rana peptides includes truncated Rana peptides lacking redundant segments of the original molecules, and protease-resistant modified Rana peptides containing D-amino acid residues and/or unnatural amino acids and/or side chain modified amino acids. The Rana peptides can also be modified by substitution of one or more of the amino acid residues valine (V), alanine (A), lysine (K), and glutamic acid (E) for the glycine (G) residues. Further modified Rana peptides include those wherein arginine (R) is substituted for one or more of the lysine (K) residues. The truncated versions of Rana peptides include the fragments cleaved on the N side of the any of the glycine residues or between repeat residues as well as N-terminally deleted fragments. Any of the Rana peptides can be modified with amino acid residues having modified side chains such as those in which the epsilon amino group of a lysine (K) residue has been coupled to a fatty acid such a palmitate or glycosylated sugar, those in which the epsilon carboxy group of aspartic (D) or glutamic (E) acid residues or a hydrolyzed glutamine (Q) residue have been coupled to fatty alcohols or glycosylated sugars, or those in which the epsilon hydroxyl group of threonine (T), tyrosine (Y), or serine (S) residues have been coupled to fatty acids or glycosylated sugars. In addition, the modified Rana peptides include those in which a truncated version is synthetically condensed with a non-natural peptide or peptide sequence optionally containing heterocyclic organic moieties such as a beta or gamma amino acid, an aliphatic

diamine, an aliphatic or aromatic dicarboxylic acid, pyridine carboxylic acid, aromatic diols and the like.

The Rana peptides and modified versions can be obtained by automated peptide synthesis using such techniques as the "Merrifeld" technique, the recombinant DNA techniques involving insertion, expression and isolation, and by extraction techniques from any source in nature (for naturally occurring Rana peptides). In addition, semi-synthetic Rana peptides can be obtained by preparation of the peptide sequence using any of the foregoing techniques and coupling the peptide sequence to the chemical moiety by esterification, amidation, Schiff base formation under appropriate side chain protection if needed. These techniques are well known in the art. For example, a Merrifeld technique is described in US Patent No. 5,049,656, the disclosure of which is incorporated herein by reference, and a recombinant technique is described at U.S. Patent Nos. 4,237,224 and 5,595,887, the disclosures of which are incorporated herein by reference. Semisynthetic coupling techniques as well as amidation and esterification of carboxylic acids (eg., for the formation of the C-terminus amides and esters) are described in "Advanced Organic Chemistry", J. March, Wiley Interscience 4th Ed., 1992. An example of an extraction technique is set forth in the following experimental example.

In regard to the recombinant techniques, the Rana peptides are present in Ranid frog skin in very high abundance and so it is not envisaged that cloning of the cDNAs encoding the peptides will present a particularly difficult challenge. Standard cloning techniques can be used. Poly(A)-rich RNA from bullfrog skin can be prepared by affinity chromatography and a constructed cDNA library. A pool of mixed nucleotides encoding appropriate regions of the Rana peptide DNA sequence such as the 19-25 region of ranatuerin 1 or analogous regions of the other Rana peptide DNA sequences can be synthesized for use as a primer in the RACE (rapid amplification of cDNA 3′ end) protocol using the polymerase chain reaction. Amplification products can be cloned into the XhoI/EcoRV restriction site of the BlueScript vector (Stratagene). The clone can be labeled

by random priming and then used to screen the cDNA to obtain a full-length clone. Positive clones can be selected and analyzed by nucleotide sequencing. This approach has been used successfully to clone the cDNAs encoding esculentin and brevinin 1E from the frog *Rana esculenta* (J. Biol. Chem. 269:11956-11961, 1994). The cDNA clone containing the genome for ranatuerin or a modified version thereof can be expressed by known recombinant techniques such as by insertion and expression in E Coli. Since the Rana peptides are antimicrobial, a preferred technique will involve triggering the culture to express after it has reached maturity.

For effective prophylactic and anti-infectious use, the Rana peptides and their modified and truncated forms as well as the compositions of the present invention may be administered either alone or in combination with a pharmaceutically-acceptable carrier, by topical, oral, anal, ocular, buccal, nasal, intramuscular, subcutaneous, intravenous, or parenteral routes. The ultimate choice of route, formulation and dose is made by the attending physician or veterinarian and is based upon the patient's or animal's unique condition. However, the usual dosage for administration to humans lies in the range of approximately 50-2000 mg. P.O. per day, and preferably in about one to four doses where the dose is based upon the activity of pure Rana peptide. The usual dosage for administration to small animals lies in the same approximate range as that for humans. For large animals, the usual dosage is higher per unit of body weight so that the dose given lies in the range of about 20 to 20,000 mg. P.O. per day. This dosage may vary somewhat with the weight of the subject (human or animal) being treated; in general, about 1-40 mg./kg. of body weight per day can be employed for humans and small animals while about 1 to 400 mg./kg. of body weight per day can be employed for large animals.

The Rana peptides of this invention can be combined with inert pharmaceutical excipients such as lactose, oil, mannitol and starch, and formulated into dosage forms such as elixirs, liquids, ointments, lotions, IV fluids, alcohol, tablets, capsules, and the like. For parenteral, intramuscular,

subcutaneous and intravenous administration, these peptides can be formulated with an inert, parenterally acceptable vehicle such as water, saline, sesame oil, ethanol buffered aqueous medium, propylene glycol and the like. For topical and oral administration, these peptides can be formulated with waxes, oils, buffered aqueous medium, and the like. These various pharmaceutical dosage forms are compounded by methods well known to the pharmacist's art.

Following the foregoing regimen, the antibacterial activity of the Rana peptides as well as the modified and truncated forms thereof likely will show inhibition action against the following strains of bacteria and fungi.

1. *Escherichia coli*

Drug-resistant strains of this "harmless" organism are responsible for twice as many cases of nosocomial infections as any other microbe, particularly hospital-acquired urinary tract infections.

2. *Enterococcus sp.*

These organisms are responsible for infection of wounds especially in elderly patients undergoing surgery. *E. faecalis* is also responsible for urinary tract infections and frequently invades heart valves and prosthetic devices of hospitalized patients.

3. *Bactereoides fragilis*

Although this Gram-Negative anaerobe is a component of the normal flora of the gut, resistant strains are responsible for wound and abdominal infections in hospitalized patients.

4. Pseudomonas aeruginosa

Infections that may lead to septicemia and pneumonia, are especially common in burn patients. The organism is frequently introduced into body

orifices by contaminated catheters and other invasive instruments and proliferates in respirators and renal dialysis units

5. Klebsiella pneumoniae

The organism readily grows in glucose solutions used for intravenous therapy leading to deaths from bacteremia. Pneumonia caused by *Klebsiella* has a 50% mortality rate in spite of treatment with existing antibiotics.

6. *Serratia marcescens*

Like *Klebsiella,* this Gram-Negative organism grows in glucose solutions and infection leads to colonization of the oropharynx of chronically ill persons leading to pneumonia.

7. *Mycobacterium tuberculosis*

Since 1985, the incidence of tuberculosis has dramatically increased in the U.S.A, due to the emergence of strains resistant to Isoniazid and Rifampin, the major drugs used in treatment. Hospital epidemics in AIDS and other immunosuppressed patients are common.

8. *Streptococcus pneumoniae*

The organism is responsible for 80% of all bacterial pneumonias and is particularly prevalent in institutions housing old people such as nursing homes and geriatric wards. In young people infection of the middle ear leads to meningitis and otitis media.

9. *Streptococcus pyrogenes*

The organism responsible for "strep throat" which, if improperly treated, can lead to complications of rheumatic heart disease, pneumonia and glomerulonephritis.

10. *Haemophilus influenzae*

This pathogen is responsible for the increasing incidence of acute bacterial meningitis in children and is often transmitted in day-care settings. Even with prompt diagnosis and aggressive treatment with existing antibiotics, 33% of children sustain residual disability.

11. *Staphylococcus saprophyticus*

Staphylococcus sp. in hospitals is generally antibiotic resistant and poses a particular problem in nurseries, delivery rooms and burn units. *S. saprophyticus* causes urinary-tract infections particularly in sexually active young women and in immunosuppressed patients.

12. *Candida albicans*

Antibiotic therapy or severe immunosuppression often precedes extensive invasion by *C. albicans* and is particularly common in burn patients and in recipients of renal, heart and bone marrow transplants.

The following experimental examples further illustrate the invention. They are not meant to provide limitations of the invention that has been fully characterized by the foregoing discussion. The foregoing specification, examples and data provide a complete description of the preparation and use of the composition of the invention. Since many embodiments of the invention can be made without departing from the spirit and scope of the invention, the invention resides in the claims hereinafter appended.

MATERIALS AND METHODS

Taxonomic identification of individual specimens of frogs of the genus *Rana is* often difficult especially in regions where several species coexist and produce hybrids. Similarly, the phylogenetic interrelationships between the different species are incompletely understood. It is suggested, therefore, that

study of the distribution and amino acid sequences of antimicrobial peptides in skin may prove to be a valuable tool for the identification and taxonomic classification of Ranid frogs. Once a larger data set of primary structures has been compiled, the amino acid sequences may form a basis for cladistic analysis to investigate phylogenetic interrelationships between species. Skin secretions, containing very high concentrations of antimicrobial peptides, can easily be collected from Ranid frogs by non-invasive procedures (mild electrical stimulation or subcutaneous injection of epinephrine) so that identification of the species does not necessitate sacrifice of the animal. Molecular techniques have been used previously to facilitate identification and to study phylogenetic relationships among Ranid frogs. For example, analysis of the nucleotide sequences of mitochondrial and genomic DNA has been used to investigate the evolutionary history of specimens of the closely related species of European water frogs, *R. ridibunda, R. esculenta* and *R. lessonae.*

Animals

Adult specimens of both sexes of the spotted frog, *R. luteiventris* (n = 16; wt range 12 - 40 g), the Rio Grande leopard frog, *R. berlandieri* (n = 8; wt range 51 - 115 g), the North American bull frog R. *catesbeiana, R. clamitans* (n=21; wt range 44-75 g), *R. grylio* (n=6; wt range 106-204g) and the northern leopard frog, *R. pipiens* (n = 12; wt range 41 - 54 g) were purchased from Charles D. Sullivan Company (Nashville, TN, U.S.A.). The animals were anaesthetized by immersion in crushed ice and were sacrificed by pithing. Skin was immediately removed, frozen on dry ice and stored at -55°C until time of extraction.

Tissue Extraction

The frozen skins (*R. luteiventris* 45 g; *R. berlandieri* 66 g; *R. clamitans* 125g; *R. grylio;* 96 g; *R. pipiens* 58 g) were separately extracted by homogenization in ethanol/0.7 M HCl (3:1 v/v; 10 ml/g) at 0°C using a Waring blender. The homogenates were stirred for 2 h at 0°C and centrifuged (4000 x g

for 30 min at 4°C). Ethanol was removed from the supernatants under reduced pressure and, after further centrifugation (4000 x g for 30 min at 4°C), the extracts were separately pumped onto 8 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, U.S.A.) connected in series at a flow rate of 2 ml/min. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried.

For the *R. catesbeiana* the tissue extraction was as follows. Skin (176 g) was removed from pithed adult specimens of *R. catesbeiana* of both sexes (n = 6) and immediately frozen on dry-ice. The tissue was stored at -55°C until time of extraction. The tissue was extracted by homogenization in ethanol/0.7 M HCl (3:1 v/v; 1800 ml) at 0 °C using a Waring blender. The homogenate was stirred for 2 h at 0 °C and centrifuged (4000 x g for 30 min at 4 °C). Ethanol was removed from the supernatant under reduced pressure and, after further centrifugation (4000 x g for 30 min at 4 °C), the extract was pumped onto 8 Sep-Pak C-18 cartridges (Waters Associates) connected in series at a flow rate of 2 ml/min. Bound material was eluted with acetonitrile/ water/ trifluoroacetic acid ((70.0:29.9:0.1, v/v/v) and freeze-dried.

Peptide Purification.

The same chromatographic procedures were used to purify antimicrobial peptides from each of the species *R. luteiventris, R. berlandieri, R. clamitans, R. grylio* and *R. pipiens* so only the methods used to isolate the *R. luteiventris* peptides will be described in detail. The skin extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid/water (5 ml) and chromatographed on a (100 x 2.6 cm) column of Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 48 ml/h and fractions (8 ml) were collected. Absorbance was measured at 280 nm. The ability of aliquots (50 µl) of the fractions to inhibit the growth of *S. aureus* was determined as described in the previous section. Fractions containing maximum activity (denoted by the bars in Fig. 1)

were pooled and injected onto a (25 x 1 cm) Vydac 218TP510 C-18 reverse-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 49% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and 280 nm and fractions (1 min) were collected. The fractions containing antimicrobial activity were successively rechromatographed on (250 x 4.6 mm) Vydac 214TP54 (C-4) and Vydac 219TP54 (phenyl) reverse-phase HPLC columns. The concentration of acetonitrile in the eluting solvent was raised from 21% to 55% over 40 min and the flow rate was 1.5 ml/min.

The purification of the *R. catesbeiana* frog skin extract was accomplished in similar fashion. After partial purification on Sep-Pak cartridges, the extract was redissolved in 1% (v/v) trifluoroacetic acid/water (5 ml) and chromatographed on a (100 x 2.6 cm) column of Sephadex G-25 (Pharmacia Biotech) equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 48 ml/h and fractions (8 ml) were collected. Absorbance was measured at 280 nm. The antimicrobial activity of aliquots of the fractions was determined. Fractions containing maximum activity (denoted by the bars in Fig. 1) were pooled and injected onto a (25 x 1 cm) Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 49% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and 280 nm and fractions (1 min) were collected. The fractions containing antimicrobial activity were successively rechromatographed on (250 x 4.6 mm) Vydac 219TP54 (phenyl) and Vydac 208TP54 (C-8) columns. The concentration of acetonitrile in the eluting solvent was raised from 14% to 35% over 40 min and the flow rate was 1.5 ml/min.

Separation of R. luteiventris peptides

The peptide fractions in the extract of *R. luteiventris* skin showing growth-inhibiting activity against *S. aureus*, after partial purification on Sep-Pak cartridges, were eluted from a Sephadex G-25 gel permeation column as two discrete zones, designated by the bars in Fig. 1. Subsequent characterization studies demonstrated that zone A contained esculentin-2L, ranatuerin-2La, and ranatuerin-2Lb and zone B contained brevinin-1La, brevinin-1Lb, temporin-1La, temporin-1Lb and temporin-1Lc. After chromatography of pooled fractions from zone A on a semipreparative Vydac C-18 reverse-phase HPLC column (Fig. 2A), the antimicrobial activity was eluted in a single, but very broad region, of the chromatogram with retention time between 45 and 70 min. Chromatography of pooled fractions from zone B (Fig. 1) on the semipreparative C-18 column resulted in the elution of the antimicrobial activity in overlapping fractions with retention times between 48 an 75 min (Fig. 2B).

The same chromatographic procedures were used to purify all the biologically active peptides and so only the purification of those from zone A (Fig. 1) will be described in detail. Fractions with retention times between 45 and 55 min from the semipreparative C-18 column (Fig. 2A) were pooled and chromatographed on a Vydac C-4 column (Fig. 3A). Although resolution was poor, antimicrobial activity (associated with ranatuerin 2La) was eluted in the fractions denoted by the bar. Fractions with retention times between 56 and 70 min from the C-18 column (Fig. 2A) were pooled and chromatographed on a Vydac C-4 column (Fig. 3B). Antimicrobial activity was eluted in two discrete zones denoted by the bars. The earlier eluting fractions contained ranatuerin--2Lb and the later eluting fractions contained esculetin-2La. The antimicrobial peptides were purified to near homogeneity, as assessed by symmetrical peak shape, by a final chromatography on a Vydac phenyl column as shown in Figs. 4 (A- C). The final yields of pure peptides were: ranatuerin 2La, 4050 nmol; ranatuerin 2Lb, 320 nmol; and esculentin-2L, 740 nmol.

Purification of antimicrobial peptide from zone B from gel permeation chromatography (Fig. 1) on Vydac C-4 and Vydac phenyl columns resulted in the isolation in pure form of brevinin-1La (880 nmol), brevinin-1lb (45 nmol), temporin-1La (120 nmol), temporin-1Lb (200 nmol) and temporin-1Lc (60 mnol). The final yields of the pure peptides are shown in parentheses.

Separation of R. berlandieri, R. clamitans, R. grylio and R. pipiens peptides

Using the same chromatographic procedures for separation of antimicrobial peptides from *R. luteiventris* led to the isolation in pure form of the following peptides from R. *berlandieri* skin in the yields indicated: brevinin-1Ba (4 nmol), brevinin-1Bb (15 nmol), brevinin-1Bc (8 nmol), brevinin-lBd (28 nmol), brevinin-1Be (12 nmol), brevinin- lBf (9 nmol), esculentin-2B (31 nmol), and ranatuerin-2B (75 nmol). The following peptides with antimicrobial activity were isolated from *R. pipiens* skin in the yields indicated: brevinin-1Pa (1050 nmol), brevinin-lPb (1110 nmol), brevinin-lPc (64 nmol), brevinin-lPd (315 nmol), brevinin-lPe (8 nmol), esculentin-2P (12 nmol), ranatuerin-2P (690 nmol), and temporin-1P (205 nmol). The following peptides were isolated from *R. clamitans* skin in the yields indicated: ranatuerin-1C (570nmol), ranalexin-1C (715 nmol), ranatuerin-2Ca (30 nmol), ranatuerin-2Cb (110 nmol), Temporin-1Ca (240 nmol), temporin-1Cb (qnm), temporin-1Cc (qnm). The following peptides were isolated from *R. grylio* skin in the yields indicated: ranalexin-1G (50 nmol), ranatuerin-1G (70 nmol), ranatuerin-2G (55 nmol), and temporin-1Ga (180 nmol). (qnm means quantity not measured.)

Separation of R. catesbeiana peptides

The peptide fractions in the extract of *R. catesbeiana* skin, after partial purification on Sep-Pak cartridges, were eluted from a Sephadex G-25 gel permeation column as two zones (Fig. 5). After chromatography of pooled fractions from the earlier-eluting zone A on a semipreparative Vydac C-18 reversed-phase HPLC column, the activity was eluted in three non-overlapping

fractions that were subsequently shown to contain ranatuerins 1+ 5, ranatuerins 2+3 and ranatuerin 4 (Fig. 6A). Similarly, chromatography of pooled fraction from zone B led to the separation of activity into three fractions subsequently shown to contain ranatuerins 6+ 7, ranatuerin 8 and rantuerin 9 (Fig. 6B). The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape, by chromatography on analytical Vydac phenyl and C-8 columns. The approximate yields of pure peptides were: ranatuerin 1, 120 nmol; ranatuerin 2, 80 nmol; ranatuerin 3, 50 nmol; ranatuerin 4, 160 nmol; ranatuerin 5, 140 nmol; ranatuerin 6, 170 nmol; ranatuerin 7, 250 nmol; ranatuerin 8, 120 nmol and ranatuerin 9, 400 nmol.

Structural analysis.

Amino acid compositions were determined by precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate using a Waters AccQ Tag system with fluorescence detection and separation of the amino acid derivatives by reverse-phase HPLC. Hydrolysis in 5.7 M HCl (24 h at 110°C) of approximately 1 nmol of peptide was carried out. The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 471 A sequenator modified for on-line detection of phenylthiohydantoin amino acids under gradient elution conditions. Electrospray mass spectrometry was carried out using a Perkin Elmer Sciex API 150EX single quadrupole instrument. The accuracy of mass determinations was ± 0.02%.

The primary structures of the peptides their amino acid sequences are shown in Table 1, Summary of the Invention. In all cases, the proposed amino acid sequences, including the presence of a cystine bridge in the brevinin-1, esculentin-2 and ranatuerin-2 peptides were confirmed by amino acid composition analysis (data not shown) and mass spectrometry (Fig. 5). Mass spectrometry also demonstrated that the peptides of the temporin family contained a C-terminally α-amidated residue.

Antimicrobial assays

Minimal inhibitory concentrations (MICs) of the peptides were determined by a standard microdilution method using 96-well microliter cell-culture plates as previously described. Serial dilutions of the peptides in Mueller-Hinton broth (50 μL) were incubated with an inoculum (50 μL of 10^3 CFU/mL) from an overnight culture of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 8325), for 18 h at 37°C in an humidified atmosphere of 5% $C0_2$ in air. Incubations with *Candida albicans (ATCC* 90028) were carried out in RPMI 1640 medium for 48 h at 35°C. After incubation, the absorbance at 550 nm of each well was determined using a M.A. Bioproducts model MA308 microliter plate reader. The MIC of each peptide was taken as the lowest concentration where no visible growth was observed. In order to monitor the validity of the assay, incubations with *E. coli and S. aureus* were carried out in parallel with increasing concentrations of the broad-spectrum antibiotic, bacitracin and incubations with *C albicans* in parallel with amphotercin B.

The abilities of the isolated peptides to inhibit the growth of the gram-positive bacterium *Staphylococcus aureus,* the gram-negative bacterium *Escherichia coli* and the yeast, *Candida albicans* are compared in Table 2 (NA in Table 2 means no activity; ND means not determined.)

Ranatuerins 1-9, tested at a concentration of 20 μg/ml, showed no detectable hemolytic activity towards human erythrocytes.

TABLE 2

MIC (μM)

	S. aureus	E. coli	C. Albicans
Rana catesbeiana			
Ranatuerin 1	50	2	70
Ranatuerin 2	60	60	NA

Ranatuerin 3	60	70	NA
Ranatuerin 4	55	ND	NA
Ranatuerin 5	>200	ND	NA
Ranatuerin 6	100	ND	NA
Ranatuerin 7	200	ND	NA
Ranatuerin 8	130	ND	NA
Ranatuerin 9	130	ND	NA
Rana luteiventris			
Brevinin-1Lb	8	16	ND
Esculetin-2L	3	6	53
Ranatuerin-2La	11	4	>150
Ranatuerin-2Lb	4	4	62
Temporin-1La	60	ND	ND
Temporin-1Lb	48	>150	>150
Temporin-1Lc	125	>150	>150

Rana berlandieri			
Brevinin-1Ba	2	ND	ND
Brevinin-1Bb	1	3	10
Brevinin-1Bc	13	ND	ND
Brevinin-1Bd	3	7	7
Brevinin-1Be	3	15	ND
Brevinin-1Bf	8	19	ND
Esculetin-2B	1	1	29
Ranatuerin-2B	1	1	35
Rana pipiens			
Brevinin-Ipa	7	14	5
Brevinin-1Pb	5	14	7
Brevinin-1Pc	7	5	7
Brevinin-1Pd	27	78	29
Esculetin-2P	ND	10	ND
Ranatuerin-2P	50	13	67
Temporin-1P	110	ND	ND
Rana clamitans			
Ranatuerin-1C	55	1.5	58
Ranalexin-1C	17	4	14
Ranatuerin-2Cb	40	2	46
Temporin-1Ca	100	NA	NA
Temporin-1Cb	160	NA	NA
Temporin-1Cc	100	NA	NA

Rana grylio			
Ranatuerin-1G	60	3.5	110
Ranatuerin-2G	150	19	>150
Ranalexin-1G	18	9	70
Temporin-1Ga	125	NA	NA
Temporin-1Gb	55	NA	NA

Structure-Activity Study of ranatuerin-1.

Substitutions in the C-terminal domain of the molecule that increase the alpha-helical character are studied. Many increase potency towards gram-negative bacteria.

Ranatuerin-1 (SMLSVLKNLG[10]KVGLGLVACK[20]INKQC) (SEQ ID NO: 8), isolated from the skin of the American bullfrog Rana catesbeiana, shows antimicrobial activity against the gram-negative bacterium, Escherichia coli, the gram-positive bacterium Staphylococcus aureus, and the yeast, Candida albicans.

The peptide is predicted to comprise three structural domains: alpha-helix (residues 1- 8), beta-sheet (residues 11- 16) and beta-turn (residues 20-25). Substitution of Cys19 and Cys25 by Ser residues had only a minor effect on potency demonstrating that the disulfide bridge is not necessary for activity. but deletion of the cyclic heptapeptide region produced an inactive analog.

Substitution of Gly10, Gly13 and Gly15 by either Lys or Glu residues produced analogs with increased a-helical content but with decreased or absent antimicrobial activity peptide thus demonstrating the importance of the central beta-sheet region of the peptide.

Substitution of Asn22 in the beta-turn region by Ala resulted in an increase in predicted alpha-helical character and an 8-fold increase in potency against E. coli.

In contrast, substitution of Ser4 in the N-terminal a-helical region by either Ala or Gly resulted in relatively minor effects on potency but deletion of residues (1-8) produced an inactive analog.

Substitution of Lys7 and Lys11 by Arg produced analogs with increased positive charge but with decreased potency. The data demonstrate that the full sequence of the peptide is necessary for biological activity and that an increase in the alpha-helical content of the C-terminal region results in an increase in activity towards a gram-negative bacterium.

Ranatuerin Analog Synthesis

All peptides were synthesized by solid-phase methodology on a 0.025 mmol scale on an Applied Biosystems model 432A peptide synthesizer using a 4-(2,4-dimethoxy-phenyl-Fmoc-aminomethyl)phenoxyacetamido-ethyl resin (Perkin Elmer, Foster City, CA). Fmoc amino acid derivatives were activated with O-benzotriazol-1-yl-N,N,N,N-tetramethyluronium hexafluorophosphate (1-equivalent), 1-hydroxybenzotriazole hydrate (1equivalent) and diisopropylethylamine (2 equivalents) . Deprotection of the N-terminus by piperidine was monitored by on-line measurement of the conductance of the carbamate salt of the Fmoc group and optimum coupling times were determined by the instrument in response to the deprotection times. The peptide was cleaved from the resin with trifluoroacetic acid/water/thioanisole/1,2-ethanedithiol (90.0/5.0/2.5/2.5) at 250 for 3 hr.

The crude synthetic peptides were purified to near homogeneity by chromatography on a 1 x 25-cm Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 20 acetonitrile/water/ trifluoroacetic acid at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and the major peak in the chromatogram was collected by hand.

The synthetic peptides were characterized by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin amino acids under gradient elution conditions and by electrospray mass spectrometry using a Perkin Elmer Sciex API 150EX single quadrupole instrument. The accuracy of mass determinations was B1 0.02%.

Antimicrobial assays

Minimal inhibitory concentrations (MICs) of the peptides were determined by a standard microdilution method using 96-well microtiter cell-culture plates as previously described. Serial dilutions of the peptides in Mueller-Hinton broth (50 mL) were incubated with an inoculum (50 mL of 103 CFU/mL) from an overnight culture of Escherichia coli (ATCC 25922) and Staphylococcus aureus (NCTC 8325), for 18 h at 37 0C in a humidified atmosphere of 5% C02 in air. Incubations with Candida albicans (ATCC 90028) were carried out in RPMI 1640 medium for 48 h at 35 0C. After incubation, the absorbance at 550 nm of each well was determined using a M.A. Bioproducts model MA308 microtiter plate reader. The MIC of each peptide was taken as the lowest concentration where no visible growth was observed. In order to monitor the validity of the assay, incubations with E. coli and S. aureus were carried out in parallel with increasing concentrations of the broad-spectrum antibiotic, bacitracin and
incubations with C. albicans in parallel with amphotercin B. The MIC's are presented in
Table 3.

TABLE 3

MIC values (μm)

Analog	*E. coli*	*S. aureus*	*C. albicans*

Analog	*E. coli*	*S. aureus*	*C. albicans*
Ranatuerin-1	3	50	70
[Ser19,Ser25]Ranatuerin-1	14	28	110
[Ala4, Ser19,Ser25]Ranatuerin-1	10	190	110
[Gly4, Ser19,Ser25]Ranatuerin-1	28	NA	140
[Arg7, Ser19,Ser25]Ranatuerin 1	14	190	190
[Arg11, Ser19,Ser25]Ranatuerin 1	28	110	110
[Lys13,Ser19,Ser25]Ranatuerein-1	190	NA	NA
[Ala22, Ser19,Ser25]Ranatuerin-1	1.5	200	110
[Lys10,Lys13Lys15-Ser19,Ser25]Ranatuerin-1	120	NA	NA
[Glu10,Glu13,Glu15-Ser19,Ser25]Ranatuerin-1	NA	NA	NA
Ranatuerin-1(1-19)fragment	NA	NA	NA
Ranatuerin-1(9-25)fragment	NA	NA	NA

NA: no activity up to 200 mM

What is claimed is:

1. An antibacterial peptide selected from any of the following Rana peptide families wherein each peptide of a family is in the form of a C-terminus carboxylic acid, a C-terminus amide or a C-terminus ester, wherein the single or multiple dashed lines of the Ranatuerin-2 family peptides signify a single bond between the two amino acid residues adjacent the dashed lines, and wherein each dashed line of the Ranatuerin-2 family peptides stands for an amino acid residue position for the purpose of aligning the amino acid residues of the peptides of that family so that the residues are homologously positioned:

Ranatuerin-1 family

Ranatuerin-1	SMLSVLKNLGKVGLGFVACKINKQC	(SEQ ID NO: 8)
Ranatuerin-1G	SMISVLKNLGKVGLGFVACKVNKQC	(SEQ ID NO: 9)
Ranatuerin-1C	SMLSVLKNLGKVGLGLVACKINKQC	(SEQ ID NO: 10)

Ranatuerin-2 family

Ranatuerin-2	GLFLDTLKGAAKD-----AGK-LEGLKCKITGCKLP	(SEQ ID NO: 12)
Ranatuerin-3	GFL-DIIKNLGKTF----AGHMLDKIKCTIGTCPPSP	(SEQ ID NO: 13)
Ranatuerin-2P	GLM DTVKNVAKNL----AGHMLDKLKCKITGC	(SEQ ID NO: 14)
Ranatuerin-2B	GLL DTIKGVAKTV----AASMLDKLKCKISGC	(SEQ ID NO: 15)
Ranatuerin-2Ca	GLFLDTLKGAAKDV----AGKLLEGLKCKIAGCKP	(SEQ ID NO: 16)
Ranatuerin-2Cb	GLFLDTLKGL--------AGKLLQGLKCIKAGCKP	(SEQ ID NO: 17)
Ranatuerin-2La	GI-LDSFKGVAKGVAKDLAGKLLDKLKCKITGC	(SEQ ID NO: 18)
Ranatuerin-2Lb	GILSSI-KGVAKGVAKNVAAQLLDTLKCKITGC	(SEQ ID NO: 19)
Ranatuerin-2G	GLLLDTLKGAAKDI----AGIALEKLKCKITGCKP	(SEQ ID NO: 20)

Esculentin-2 family

Esculentin-2L	GILSLFTGGIKALGKTLFKMAGKAGAEHLACKATNQC	(SEQ ID NO: 22)

Esculentin-2B GLFSILRGAAKFASKGLGKDLTKLGVDLVACKISKQC (SEQ ID NO: 23)
Esculentin-2P GFSSIFRGVAKFASKGLGKDLARLGVNLVACKISKQC (SEQ ID NO: 24)

Brevinin-1 family

Ranatuerin-4	FLPFIARLAAKVFPSIICSVTKKC	(SEQ ID NO: 26)
Brevinin-lLa	FLPMLAGLAASMVPKLVCLITKKC	(SEQ ID NO: 27)
Brevinin-lLb	FLPMLAGLAASMVPKFVCLITKKC	(SEQ ID NO: 28)
Brevinin-lBa	FLPAIAGMAAKFLPKIFCAISKKC	(SEQ ID NO: 29)
Brevinin-lBb	FLPAIAGMAAKFLPKIFCAISKKC	(SEQ ID NO: 30)
Brevinin-lBc	FLPFIAGVAAKFLPKIFCAISKKC	(SEQ ID NO: 31)
Brevinin-lBd	FLPAIAGVAAKFLPKIFCAISKKC	(SEQ ID NO: 32)
Brevinin-lBe	FLPAIVGAAAKFLPKIFCVISKKC	(SEQ ID NO: 33)
Brevinin-lBf	FLPFIAGMAANFLPKIFCAISKKC	(SEQ ID NO: 34)
Brevinin-lPa	FLPIIAGVAAKVFPKIFCAISKKC	(SEQ ID NO: 35)
Brevinin-lPb	FLPIIAGIAAKVFPKIFCAISKKC	(SEQ ID NO: 36)
Brevinin-lPc	FLPIIASVAAKVFSKIFCAISKKC	(SEQ ID NO: 37)
Brevinin-lPd	FLPIIASVAANVFSKIFCAISKKC	(SEQ ID NO: 38)
Brevinin-lPe	FLPIIASVAAKVFPKIFCAISKKC	(SEQ ID NO: 39)

Ranalexin-1 family

Ranalexin-lC	FLGGLMKAFPALICAVTKKC	(SEQ ID NO: 41)
Ranalexin-1G	FLGGLMKIIPAAFCAVTKKC	(SEQ ID NO: 42)

Temporin-A family **wherein the C-terminus is a simple carboxamide**

Ranatuerin 5	FLPIASLLGKYL	(SEQ ID NO: 44)
Ranatuerin 6	FISAIASMLGKFL	(SEQ ID NO: 45)
Ranatuerin 7	FLSAIASMLGKFL	(SEQ ID NO: 46)
Ranatuerin 8	FISAIASFLGKFL	(SEQ ID NO: 47)
Ranatuerin 9	FLFPLITSFLSKVL	(SEQ ID NO: 48)
Temporin-lCa	FLPFLATLLSKVL	(SEQ ID NO: 49)
Temporin-lCb	FLPFLAKILTGVL	(SEQ ID NO: 50)
Temporin-lCc	FLPLPASLFGKKL	(SEQ ID NO: 51)
Temporin-lPa	FLPIVGKLLSGLL	(SEQ ID NO: 52)

Temporin-lLc	FLPILINLIHKGLL	(SEQ ID NO: 53)

Temporin-B Family **wherein the C-terminus is a simple carboxamide**

Temporin-lLa	VLPLISMALGKLL	(SEQ ID NO: 55)
Temporin-lLb	NFLGTLINLAKKIM	(SEQ ID NO: 56)
Temporin-lGa	SILPTIVSFLSKFV	(SEQ ID NO: 57)
Temporin-lGb	SILPTIVSFLSKFL	(SEQ ID NO: 58)
Temporin-lGc	SILPTIVSFLTKFIL	(SEQ ID NO: 59)
Temporin-lGd	FIIPLIASFLSKFIL	(SEQ ID NO: 60)

2. A modified Rana peptide of any of the following formulas wherein each formula corresponds to a single Rana peptide family of claim 1 and wherein the symbols X, B, Z, U, O, J' and J and their position/location within a formula designate either any of the amino acid residues occurring at the same position within the other peptides of the same family or a conservative substitution of an amino acid residue for the amino acid substitution of an amino acid residue at the same position for any of the peptides within the same family:

(i) a Ranatuerin-1 family modified Rana peptide of the formula SMXSVLKNLGKVGLGXVACKXNKQC (SEQ ID NO: 11) having the designation Ranatuerin-1 mod;

(ii) a Ranatuerin-2 family modified Rana peptide of the formula GBBBBBBKBBBBBBBBBBABBBBBBBKCBBBBCBBB (SEQ ID NO: 21) and having the designation Ranatuerin-2 mod;

(iii) an Esculentin-2 family modified Rana peptide of the formula GZZSZZZGZZKZZZKZLZKZZZZZGZZZZACKZZZQC (SEQ ID NO: 22) and having the designation Esculentin-2 mod;

(iv) a Brevinin-1 family modified Rana peptide of the formula FLPUUUUUAAUUUUUUUCUUUKKC (SEQ ID NO: 40) and having the designation Brevinin-1mod;

(v) a Ranalexin-1 family modified Rana peptide of the formula FLGGLMKJ'J'PAJ'J'CAVTKKC (SEQ ID NO: 43) and having the designation Ranalexin mod;

(vi) a Temporin-A family modified Rana peptide of the formula FOOOOOOOOOOOLO (SEQ ID NO: 54) and having the designation Temporin-A mod;

(vii) a Temporin-B family modified Rana peptide of the formula JJJJJIJSFLJKFJL (SEQ ID NO: 61) and having the designation Temporin-B mod.

3. A modified Rana peptide having the amino acid sequence of any of the Rana peptides of claim 1 wherein one or more of the following amino acid substitutions have been made:

a) alanine for cysteine;

b) serine for cysteine;

c) alanine for glycine;

d) valine for glycine;

e) lysine for glycine;

f) glutamine for glycine;

g) arginine for lysine;

h) alanine for asparagine

i) coupling a C_{10} to C_{20} fatty acid to the lysine side chain .

4. A truncated Rana peptide of claim 1.

5. A composition comprising a purified, antibacterial extract of the skin of a mature Ranid frog of the species selected from catesbeiana, luteiventris, berlandieri, grylio, clamitans and pipiens.

6. A pharmaceutical composition comprising an effective antibacterial amount of a Rana peptide of claim 1 and a suitable pharmaceutical carrier.

7. A pharmaceutical composition comprising an effective antibacterial amount of a modified Rana peptide of claim 2 and a suitable pharmaceutical carrier.

8. A pharmaceutical composition comprising an effective antibacterial amount of a truncated Rana peptide of claim 3 and a suitable pharmaceutical carrier.

9. A method of treatment of a bacterial infection in a patient comprising administering to the patient an antibacterially effective amount of a Rana peptide of claim 1, a modified Rana peptide of claim 2 or 3, or a truncated Rana peptide of claim 4, or a composition of claim 5, or a pharmaceutical composition of claim 6, 7 or 8.

10. A Rana peptide of claim 1 or 2 wherein the peptide is the amide or ester, the amide being a simple amide or an amide of a C1 to C10 aliphatic or aromatic primary, secondary or tertiary amine, and the ester being an ester of a C1 to C10 aliphatic or aromatic alcohol.

11. A Rana peptide of claim 1 selected from the Temporin A or Temporin B family wherein the C-terminus is a carboxylic acid.

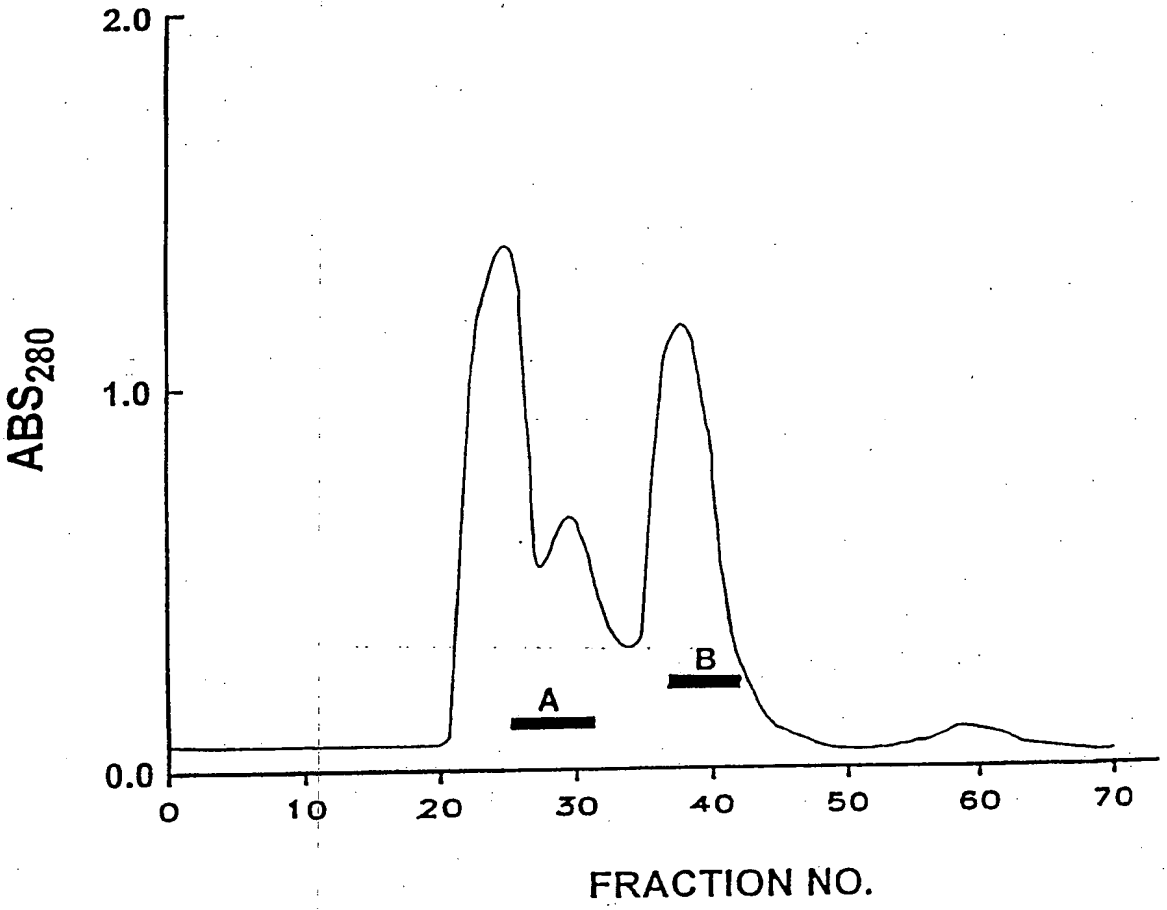

Fig. 1

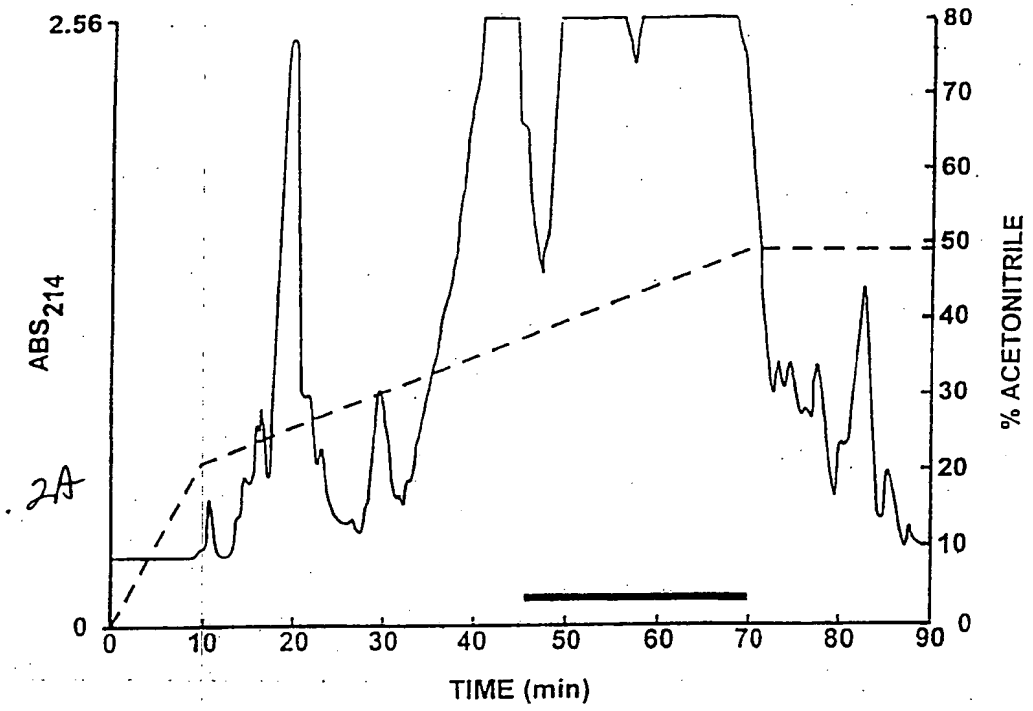
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FIG. 2A

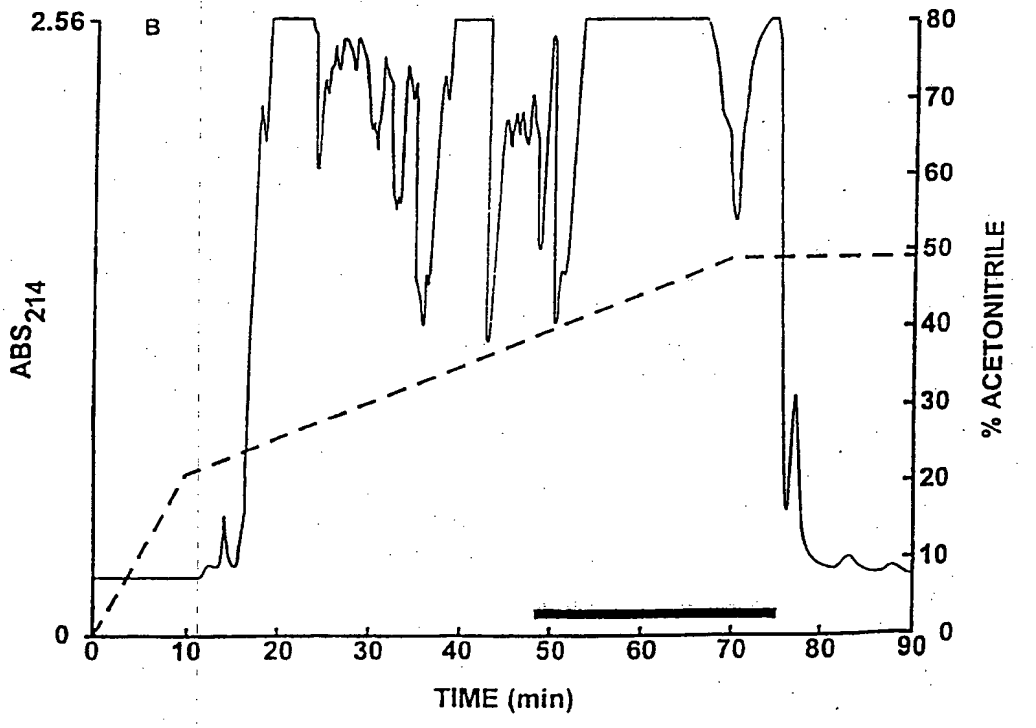
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Fig. 2B

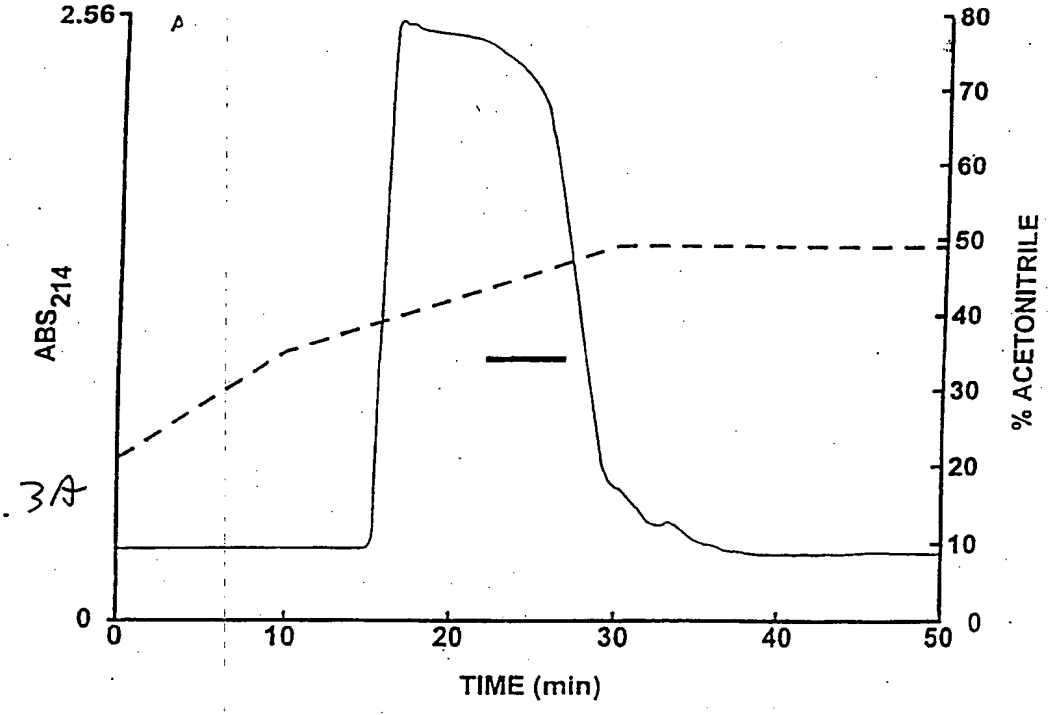
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FIG. 3A

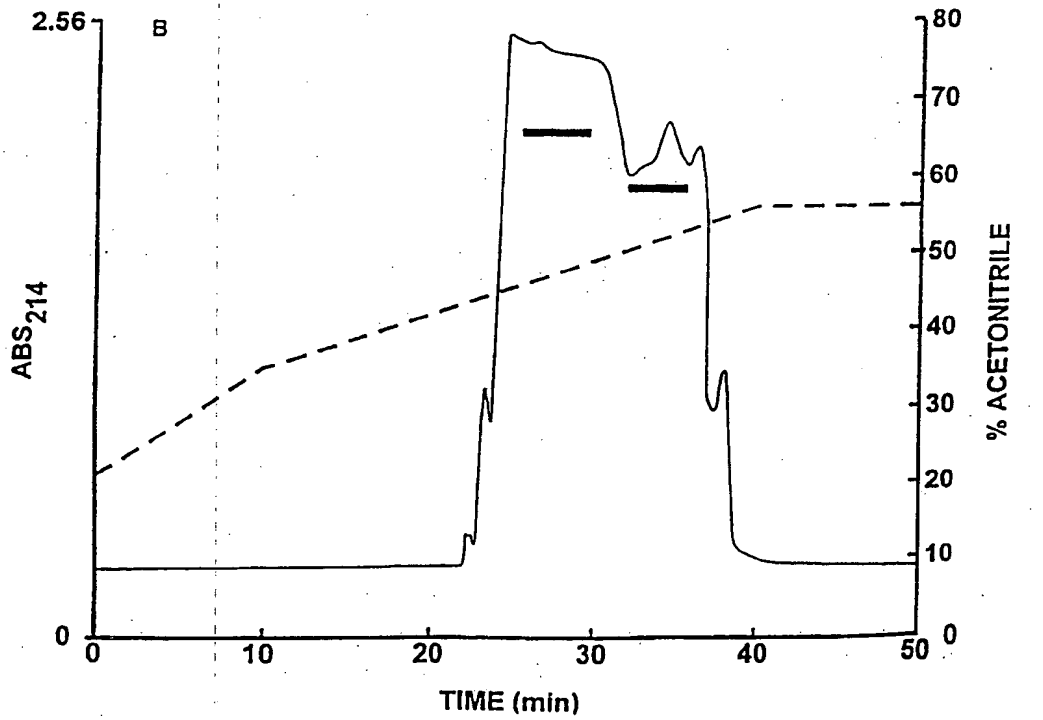
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FIG. 3B

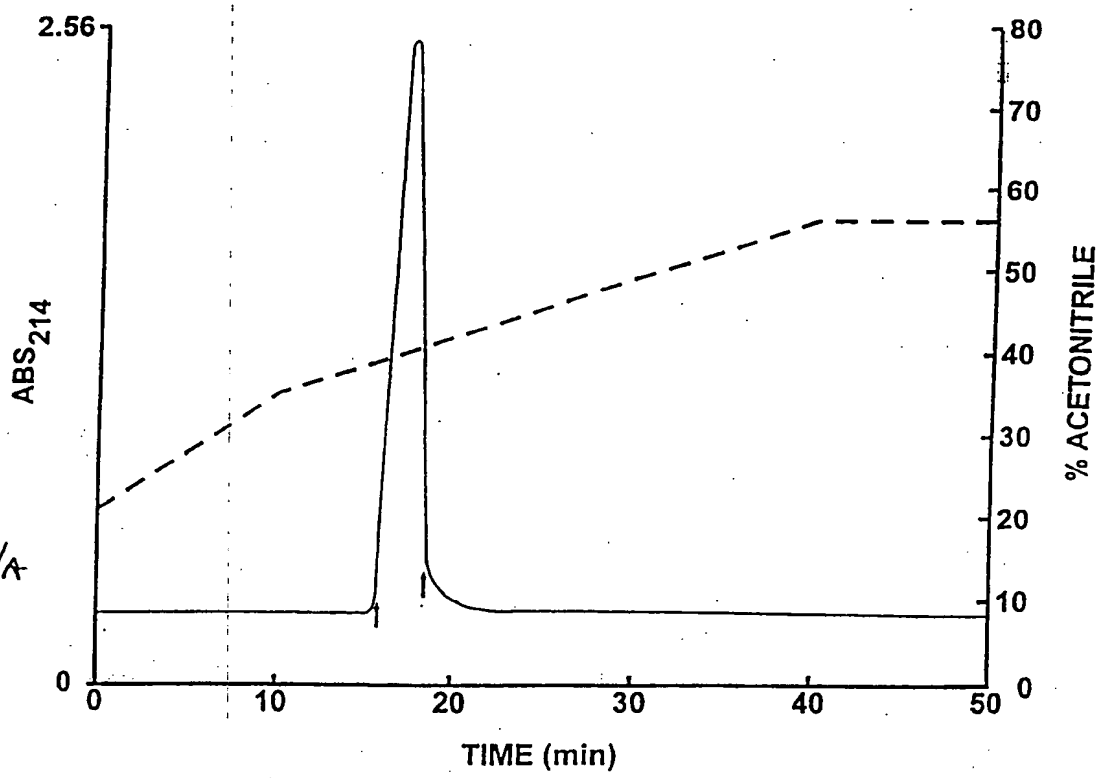
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Fig. 4A

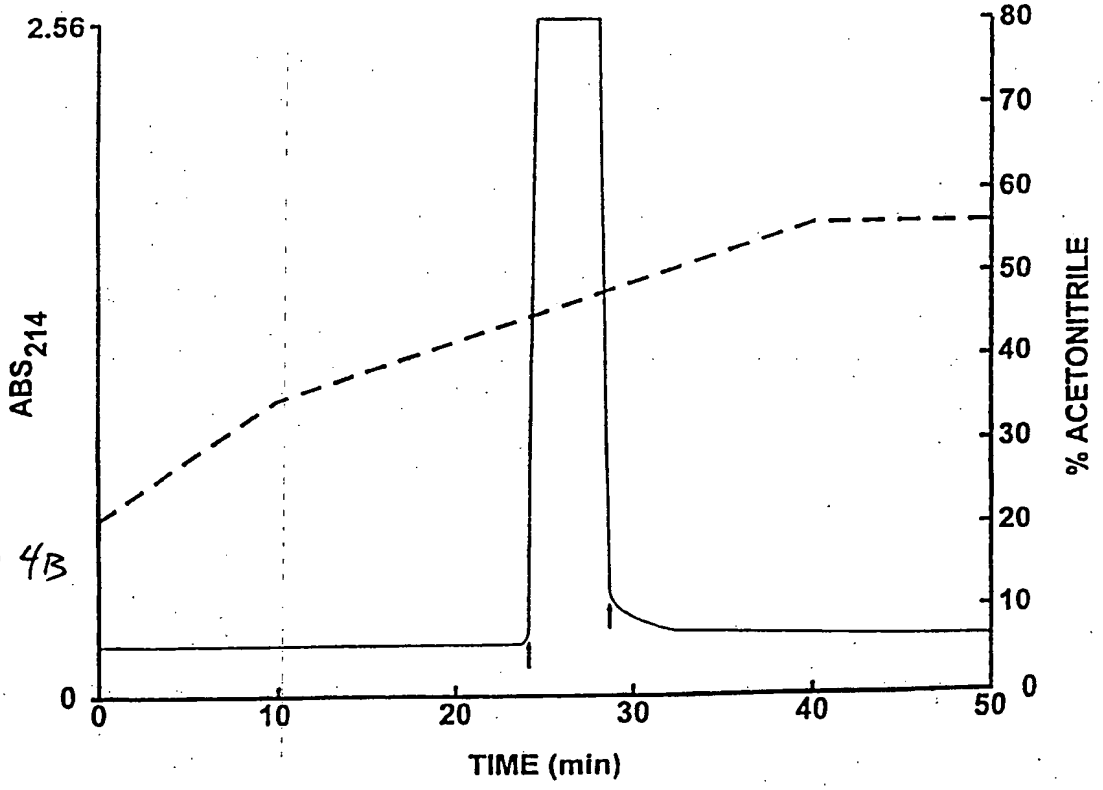
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Fig 4B

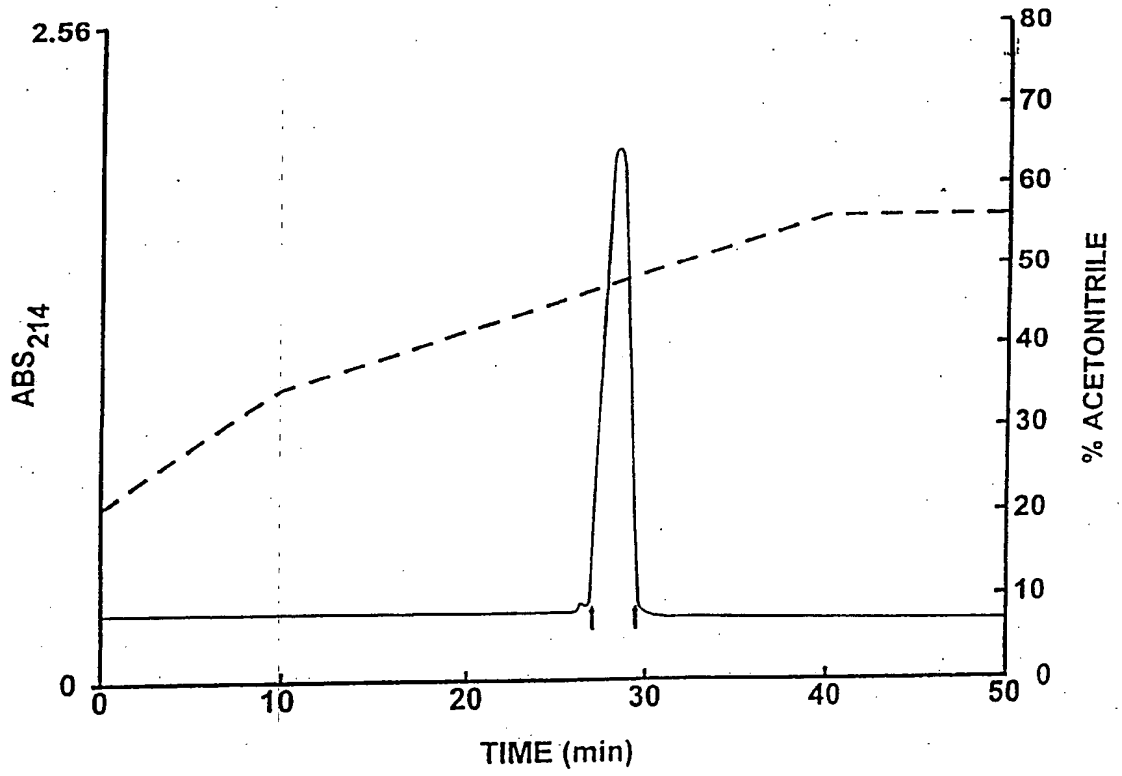
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Fig. 4e

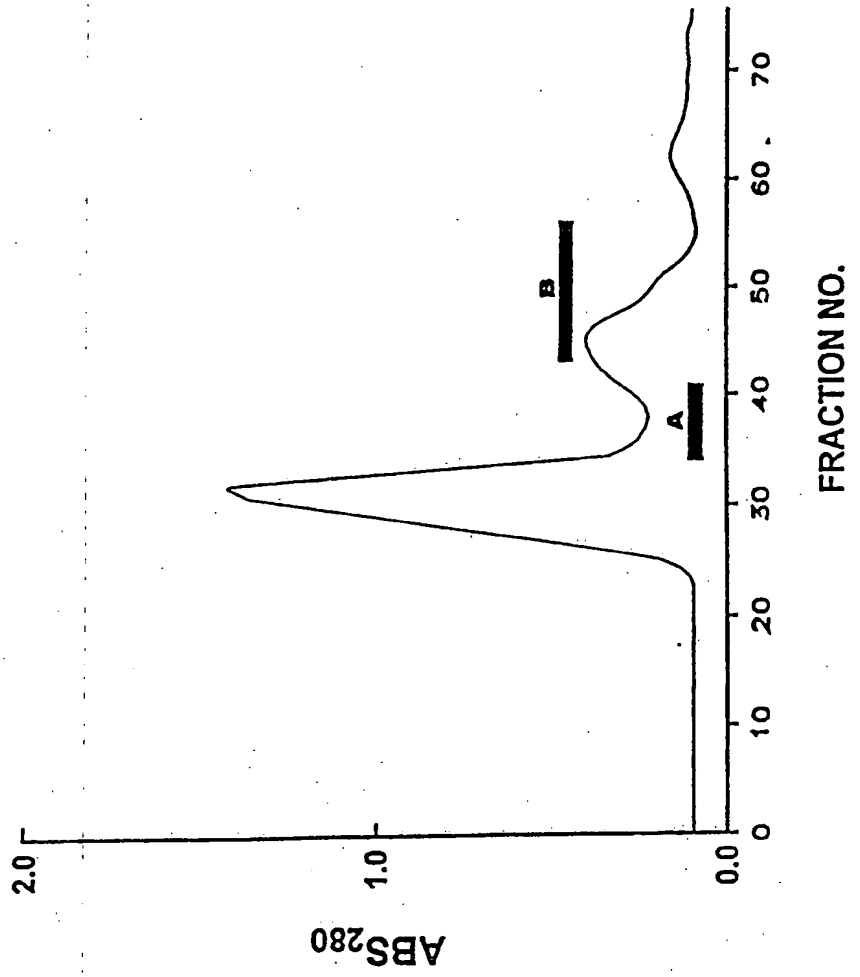

Fig. 5

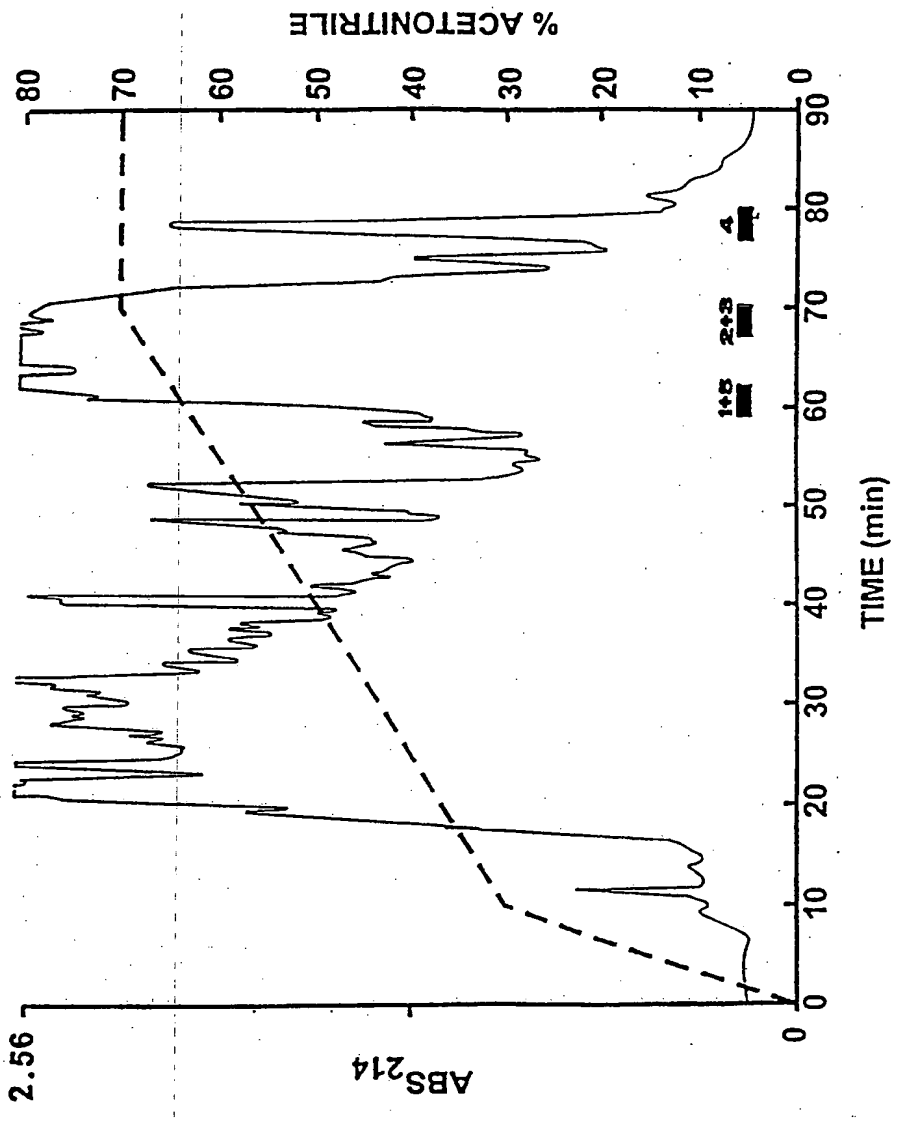
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Fig. 6A

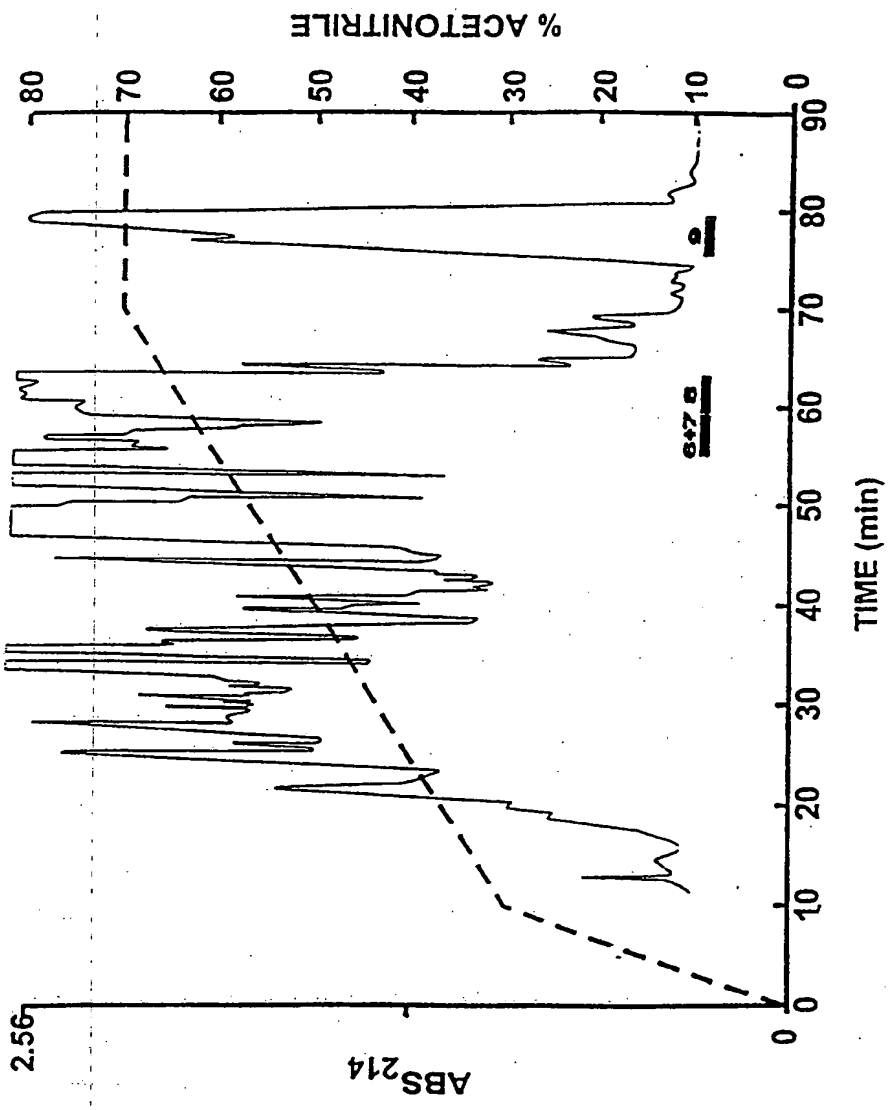
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Fig. 6B

SEQUENCE LISTING

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Gly Leu Met Asp Thr Val Lys Asn Val Ala Lys Asn Leu Ala Gly His
1 5 10 15
Met Leu Asp Lys Leu Lys Cys Lys Ile Thr Gly Cys
20 25

<210> 15
<211> 28
<212> PRT
<213> Rana berlandieri

<400> 15
Gly Leu Leu Asp Thr Ile Lys Gly Val Ala Lys Thr Val Ala Ala Ser
1 5 10 15
Met Leu Asp Lys Leu Lys Cys Lys Ile Ser Gly Cys
20 25

<210> 16

<211> 31
<212> PRT
<213> Rana clamitans

<400> 16
Gly Leu Phe Leu Asp Thr Leu Lys Gly Ala Ala Lys Asp Val Ala Gly
1 5 10 15
Lys Leu Leu Glu Gly Leu Lys Cys Lys Ile Ala Gly Cys Lys Pro
20 25 30

<210> 17
<211> 27
<212> PRT
<213> Rana clamitans

<400> 17
Gly Leu Phe Leu Asp Thr Leu Lys Gly Leu Ala Gly Lys Leu Leu Gln
1 5 10 15
Gly Leu Lys Cys Ile Lys Ala Gly Cys Lys Pro
20 25

<210> 18
<211> 32
<212> PRT
<213> Rana luteiventris

<400> 18
Gly Ile Leu Asp Ser Phe Lys Gly Val Ala Lys Gly Val Ala Lys Asp
1 5 10 15
Leu Ala Gly Lys Leu Leu Asp Lys Leu Lys Cys Lys Ile Thr Gly Cys
20 25 30

<210> 19
<211> 32
<212> PRT
<213> Rana luteiventris

<400> 19
Gly Ile Leu Ser Ser Ile Lys Gly Val Ala Lys Gly Val Ala Lys Asn
1 5 10 15
Val Ala Ala Gln Leu Leu Asp Thr Leu Lys Cys Lys Ile Thr Gly Cys
20 25 30

<210> 20
<211> 31
<212> PRT
<213> Rana grylio

<400> 20
Gly Leu Leu Leu Asp Thr Leu Lys Gly Ala Ala Lys Asp Ile Ala Gly
1 5 10 15
Ile Ala Leu Glu Lys Leu Lys Cys Lys Ile Thr Gly Cys Lys Pro
20 25 30

<210> 21
<211> 36
<212> PRT
<213> Rana catesbeiana

<220>
<221> MUTAGEN

<222> 1....36
<223> Xaa = B = Any amino acid residue in same residue position of other family members arranged homologously

<400> 21
Gly Xaa Xaa Xaa Xaa Xaa Xaa Lys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15
Xaa Xaa Ala Xaa Xaa Xaa Xaa Xaa Xaa Xaa Lys Cys Xaa Xaa Xaa Xaa
20 25 30
Cys Xaa Xaa Xaa
35

<210> 22
<211> 37
<212> PRT
<213> Rana luteiventris

<400> 22
Gly Ile Leu Ser Leu Phe Thr Gly Gly Ile Lys Ala Leu Gly Lys Thr
1 5 10 15
Leu Phe Lys Met Ala Gly Lys Ala Gly Ala Glu His Leu Ala Cys Lys
20 25 30
Ala Thr Asn Gln Cys
35

<210> 23
<211> 37
<212> PRT
<213> Rana berlandieri

<400> 23
Gly Leu Phe Ser Ile Leu Arg Gly Ala Ala Lys Phe Ala Ser Lys Gly
1 5 10 15
Leu Gly Lys Asp Leu Thr Lys Leu Gly Val Asp Leu Val Ala Cys Lys
20 25 30
Ile Ser Lys Gln Cys
35

<210> 24
<211> 37
<212> PRT
<213> Rana pipiens

<400> 24
Gly Phe Ser Ser Ile Phe Arg Gly Val Ala Lys Phe Ala Ser Lys Gly
1 5 10 15
Leu Gly Lys Asp Leu Ala Arg Leu Gly Val Asn Leu Val Ala Cys Lys
20 25 30
Ile Ser Lys Gln Cys
35

<210> 25
<211> 37
<212> PRT
<213> Rana catesbeiana

<220>
<221> MUTAGEN
<222> 1....37
<223> Xaa = Z = Any amino acid residue in same residue position of other family members arranged homologously

<400> 25
Gly Xaa Xaa Ser Xaa Xaa Xaa Gly Xaa Xaa Lys Xaa Xaa Xaa Lys Xaa
1 5 10 15
Leu Xaa Lys Xaa Xaa Xaa Xaa Xaa Gly Xaa Xaa Xaa Xaa Ala Cys Lys
20 25 30
Xaa Xaa Xaa Gln Cys
35

<210> 26
<211> 24
<212> PRT
<213> Rana catesbeiana

<400> 26
Phe Leu Pro Phe Ile Ala Arg Leu Ala Ala Lys Val Phe Pro Ser Ile
1 5 10 15
Ile Cys Ser Val Thr Lys Lys Cys
20

<210> 27
<211> 24
<212> PRT
<213> Rana luteiventris

<400> 27
Phe Leu Pro Met Leu Ala Gly Leu Ala Ala Ser Met Val Pro Lys Leu
1 5 10 15
Val Cys Leu Ile Thr Lys Lys Cys
20

<210> 28
<211> 24
<212> PRT
<213> Rana luteiventris

<400> 28
Phe Leu Pro Met Leu Ala Gly Leu Ala Ala Ser Met Val Pro Lys Phe
1 5 10 15
Val Cys Leu Ile Thr Lys Lys Cys
20

<210> 29
<211> 24
<212> PRT
<213> Rana berlandieri

<400> 29
Phe Leu Pro Ala Ile Ala Gly Met Ala Ala Lys Phe Leu Pro Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 30
<211> 24
<212> PRT
<213> Rana berlandieri

<400> 30
Phe Leu Pro Ala Ile Ala Gly Met Ala Ala Lys Phe Leu Pro Lys Ile
1 5 10 15

Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 31
<211> 24
<212> PRT
<213> Rana berlandieri

<400> 31
Phe Leu Pro Phe Ile Ala Gly Val Ala Ala Lys Phe Leu Pro Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 32
<211> 24
<212> PRT
<213> Rana berlandieri

<400> 32
Phe Leu Pro Ala Ile Ala Gly Val Ala Ala Lys Phe Leu Pro Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 33
<211> 24
<212> PRT
<213> Rana berlandieri

<400> 33
Phe Leu Pro Ala Ile Val Gly Ala Ala Ala Lys Phe Leu Pro Lys Ile
1 5 10 15
Phe Cys Val Ile Ser Lys Lys Cys
20

<210> 34
<211> 24
<212> PRT
<213> Rana berlandieri

<400> 34
Phe Leu Pro Phe Ile Ala Gly Met Ala Ala Asn Phe Leu Pro Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 35
<211> 24
<212> PRT
<213> Rana pipiens

<400> 35
Phe Leu Pro Ile Ile Ala Gly Val Ala Ala Lys Val Phe Pro Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 36
<211> 24
<212> PRT

<213> Rana pipiens

<400> 36
Phe Leu Pro Ile Ile Ala Gly Ile Ala Ala Lys Val Phe Pro Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 37
<211> 24
<212> PRT
<213> Rana pipiens

<400> 37
Phe Leu Pro Ile Ile Ala Ser Val Ala Ala Lys Val Phe Ser Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 38
<211> 24
<212> PRT
<213> Rana pipiens

<400> 38
Phe Leu Pro Ile Ile Ala Ser Val Ala Ala Asn Val Phe Ser Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 39
<211> 24
<212> PRT
<213> Rana pipiens

<400> 39
Phe Leu Pro Ile Ile Ala Ser Val Ala Ala Lys Val Phe Pro Lys Ile
1 5 10 15
Phe Cys Ala Ile Ser Lys Lys Cys
20

<210> 40
<211> 24
<212> PRT
<213> Rana catesbeiana

<220>
<221> MUTAGEN
<222> 1....24
<223> Xaa = U = Any amino acid residue in same residue position of other family members arranged homologously

<400> 40
Phe Leu Pro Xaa Ile Xaa Gly Xaa Ala Ala Xaa Xaa Xaa Pro Xaa Xaa
1 5 10 15
Xaa Cys Xaa Ile Xaa Lys Lys Cys
20

<210> 41
<211> 20
<212> PRT

<213> Rana grylio

<400> 41
Phe Leu Gly Gly Leu Met Lys Ala Phe Pro Ala Leu Ile Cys Ala Val
1 5 10 15
Thr Lys Lys Cys
20

<210> 42
<211> 20
<212> PRT
<213> Rana clamitans

<400> 42
Phe Leu Gly Gly Leu Met Lys Ala Phe Pro Ala Leu Ile Cys Ala Val
1 5 10 15
Thr Lys Lys Cys
20

<210> 43
<211> 20
<212> PRT
<213> Rana rugosa

<220>
<221> MUTAGEN
<222> 1....20
<223> Xaa = J = Any amino acid residue in same residue position of other family members arranged homologously

<400> 43
Phe Leu Gly Gly Leu Met Lys Xaa Xaa Pro Ala Xaa Xaa Cys Ala Val
1 5 10 15
Thr Lys Lys Cys
20

<210> 44
<211> 12
<212> PRT
<213> Rana catesbeiana

<400> 44
Phe Leu Pro Ile Ala Ser Leu Leu Gly Lys Tyr Leu
1 5 10

<210> 45
<211> 13
<212> PRT
<213> Rana catesbeiana

<400> 45
Phe Ile Ser Ala Ile Ala Ser Met Leu Gly Lys Phe Leu
1 5 10

<210> 46
<211> 13
<212> PRT
<213> Rana catesbeiana

<400> 46
Phe Leu Ser Ala Ile Ala Ser Met Leu Gly Lys Phe Leu

1 5 10

<210> 47
<211> 13
<212> PRT
<213> Rana catesbeiana

<400> 47
Phe Ile Ser Ala Ile Ala Ser Phe Leu Gly Lys Phe Leu
1 5 10

<210> 48
<211> 14
<212> PRT
<213> Rana catesbeiana

<400> 48
Phe Leu Phe Pro Leu Ile Thr Ser Phe Leu Ser Lys Val Leu
1 5 10

<210> 49
<211> 13
<212> PRT
<213> Rana clamitans

<400> 49
Phe Leu Pro Phe Leu Ala Thr Leu Leu Ser Lys Val Leu
1 5 10

<210> 50
<211> 13
<212> PRT
<213> Rana clamitans

<400> 50
Phe Leu Pro Phe Leu Ala Lys Ile Leu Thr Gly Val Leu
1 5 10

<210> 51
<211> 13
<212> PRT
<213> Rana clamitans

<400> 51
Phe Leu Pro Leu Phe Ala Ser Leu Ile Gly Lys Lys Leu
1 5 10

<210> 52
<211> 13
<212> PRT
<213> Rana pipiens

<400> 52
Phe Leu Pro Ile Val Gly Lys Leu Leu Ser Gly Leu Leu
1 5 10

<210> 53
<211> 14
<212> PRT
<213> Rana luteiventris

<400> 53
Phe Leu Pro Ile Leu Ile Asn Leu Ile His Lys Gly Leu Leu
1 5 10

<210> 54
<211> 14
<212> PRT
<213> Rana catesbeiana

<220>
<221> MUTAGEN
<222> 1....14
<223> Xaa = O = Any amino acid residue in same residue position of other family members arranged homologously

<400> 54
Phe Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa
1 5 10

<210> 55
<211> 13
<212> PRT
<213> Rana luteiventris

<400> 55
Val Leu Pro Leu Ile Ser Met Ala Leu Gly Lys Leu Leu
1 5 10

<210> 56
<211> 14
<212> PRT
<213> Rana luteiventris

<400> 56
Asn Phe Leu Gly Thr Leu Ile Asn Leu Ala Lys Lys Ile Met
1 5 10

<210> 57
<211> 14
<212> PRT
<213> Rana grylio

<400> 57
Ser Ile Leu Pro Thr Ile Val Ser Phe Leu Ser Lys Phe Val
1 5 10

<210> 58
<211> 14
<212> PRT
<213> Rana grylio

<400> 58
Ser Ile Leu Pro Thr Ile Val Ser Phe Leu Ser Lys Phe Leu
1 5 10

<210> 59
<211> 15
<212> PRT
<213> Rana grylio

<400> 59
Ser Ile Leu Pro Thr Ile Val Ser Phe Leu Thr Lys Phe Ile Leu

1 5 10 15

<210> 60
<211> 15
<212> PRT
<213> Rana grylio

<400> 60
Phe Ile Ile Pro Leu Ile Ala Ser Phe Leu Ser Lys Phe Ile Leu
1 5 10 15

<210> 61
<211> 15
<212> PRT
<213> Rana catesbeiana

<220>
<221> MUTAGEN
<222> 1....15
<223> Xaa = J = Any amino acid residue in same residue position of other family members arranged homologously

<400> 61
Xaa Xaa Xaa Xaa Xaa Ile Xaa Ser Phe Leu Xaa Lys Xaa Xaa Leu
1 5 10

<210> 62
<211> 25
<212> PRT
<213> Unknown

<220>
<221> VARIANT
<222> 1....25
<223> Model sequence for structure activity relationship determination

<400> 62
Ser Met Leu Ser Val Leu Lys Asn Leu Gly Lys Val Gly Leu Gly Phe
1 5 10 15
Val Ala Cys Lys Ile Asn Lys Gln Cys
20 25

<210> 63
<211> 25
<212> PRT
<213> Unknown

<220>
<221> VARIANT
<222> 1....25
<223> Model sequence for structure activity relationship determination

<400> 63
Ser Met Leu Ser Val Leu Lys Asn Leu Gly Lys Val Gly Leu Gly Phe
1 5 10 15
Val Ala Cys Lys Ile Asn Lys Gln Cys
20 25